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lutea. It is unclear whether the other gene is expressed at another tissue site or whether it represents a pseudo-gene. The fact that H2 relaxin is synthesized and expressed in the ovary suggests that this is the sequence that is directly involved in the physiology of pregnancy.

Naturally occurring relaxin is synthesized as a single-chain 23 kDa preprorelaxin with the overall structure: signal peptide, B-chain, connecting C-peptide, and A-chain. During the biosynthesis of relaxin, the signal peptide is removed as the nascent chain is moved across the endoplasmic reticulum producing the 19-kDa prorelaxin (Reddy et al., *Supra*). Further processing of the prorelaxin to relaxin occurs in vivo through the endoproteolytic cleavage of the C-peptide at specific pairs of basic amino acid residues located at the B/C-chain and A-/C-chain junctions after the formation of disulfide bridges between the B- and A-chains (Marriott et al. Mol. Endo. vol. 6 no. 9 [1992]) in a manner analogous to insulin. The relaxin disulfide bridges occur between the cysteines at A9-B10 and A22-B22 with an intra-chain disulfide bridge within the A-chain between A8 and A13 (U.S. Patent 4,656,249, issued Apr. 7, 1987).

A concise review of the knowledge about relaxin as of 1988 was provided by Sherwood, D. in The Physiology of Reproduction Chapter 16, "Relaxin", Knobil, E. and Neill, J. et al., (eds.) Raven Press, Ltd. New York pp. 585-673 [1988]. Relaxin has been consistently associated with the condition of pregnancy, and most of its known utilities are associated with this condition.

H2 relaxin has been described to remodel the reproductive tract to facilitate the birth process, including ripening of the cervix, thickening of the endometrium of the pregnant uterus as well as increased vascularization to this area, and an effect on collagen synthesis. H2 relaxin has also been associated with lactation, and some reports indicate that relaxin has a growth-promoting effect on mammary tissue (Wright, L.C. and Anderson, R.R., Adv. Exp. Med. Biol. vol 341 [1982]). Given the effect of relaxin on the connective tissue, it has been suggested that relaxin may

PROCESS FOR PRODUCING RELAXIN

Field of the Invention

This invention relates to non-naturally occurring forms
5 of prorelaxin and to a process for producing relaxin from
such a non-naturally occurring form of prorelaxin.

Background of the Invention

Mature human relaxin is an ovarian hormonal peptide of
10 approximately 6000 daltons in molecular weight known to be
responsible for remodeling the reproductive tract before
parturition, thus facilitating the birth process. The
protein appears to modulate the restructuring of connective
tissues in target organs to obtain the required changes in
15 organ structure during pregnancy and parturition. Some of
the important roles for relaxin as a pregnancy hormone
include inhibition of premature labor, cervical ripening at
parturition, and development of the mammary gland [Reddy et
al., Arch. Biochem. Biophys. 294, 579 (1992)]. While
20 predominantly a hormone of pregnancy, relaxin has also been
detected in the non-pregnant female as well as in the male
(seminal fluid).

The amino acid sequences of relaxin have been determined
by direct protein sequencing or deduced from the nucleotide
25 sequences of the DNAs for a number of species including pig,
rat [Hudson, et al. Nature 291, 127 (1981)], sand tiger
shark, spiny dogfish, skate, whale, monkey and human.
[Hudson et al. EMBO J. 3, 2333 (1984)]

Recombinant techniques were first applied to the
30 isolation of cDNA clones for rat and porcine relaxins
(Hudson, et al., Nature vol. 291, pg. 544 [1981]; Haley et
al., DNA vol. 1, pg. 155 [1982]). Two human gene forms have
been identified by genomic cloning using probes from the
porcine relaxin gene (Hudson et al., Nature vol. 301, pg. 628
35 [1983]; Hudson et al., EMBO J. vol. 3 pg 2333 [1984]; U.S.
Patent Nos. 4,758,516 [issued 19 July 1988] and 4,871,670
[issued 3 October 1989], although only one of these gene
forms (termed H2) has been found to be transcribed in corpora

improve skin elasticity. U.S. Patent 5,166,191, issued 21-Feb-1992, describes the use of relaxin in cardiovascular therapy.

U.S. Patent 5,023,321, issued June 11, 1991, discloses
5 the preparation of human preprorelaxin and sub-units thereof. U.S. Patent 4,871,670, issued October 3, 1989, discloses genes and DNA transfer vectors for the expression of human preprorelaxin and sub-units thereof.

European Pat. Publ. Nos. 101,309 published Feb. 22, 1984
10 and 112,149 published June 27, 1984 respectively disclose the molecular cloning and characterization of a gene sequence coding for human relaxin and human H2-relaxin and analogs thereof.

U.S. Patent 4,565,249, issued April 7, 1987, discloses
15 a method for the synthesis of porcine relaxin or modified forms or analogues thereof. Australian Pat. No. 561,670 issued Aug. 26, 1987, and Haley et al., DNA 1:155-162 (1982) disclose how to prepare porcine relaxin and Stewart et al., NAR vol. 11, no. 19 pg. 6597-6609 (1983) disclose expression
20 of porcine prorelaxin in E.coli. Reddy et al., Supra, disclose a method for purification of a recombinant porcine prorelaxin expressed in E.coli.

Gold et al., (Abstr. Pap. Chem. Soc. 203 Meet., Pt. 3, BTEC55 [1992]) disclose a method for the production of
25 relaxin based on the A-chain B-chain combination reaction. The A-chain was expressed in E.coli as a modified prorelaxin in refractile bodies; the A-chain was purified from the modified prorelaxin by chemical cleavage. The B-chain was produced in a second E.coli fermentation in which it was
30 secreted and subsequently purified. The two purified chains were then combined in an oxidative combination and folding reaction. During the reaction, 1 intrachain disulfide and 2 interchain disulfide bonds were formed. The two-chain combination process involves numerous process steps and the
35 use of dual fermentations for production of the two chains.

Marriott et al., [Molecular Endocrinology 6, 1441 (1992)] disclose mammalian expression of a prorelaxin variant

having non-naturally occurring cleavage sites at the A-/C-chain and B-/C-chain junctions.

Recombinant Expression

5 There exists a need for a process for producing relaxin from prorelaxin that does not require the use of dual fermentation and numerous process steps as is used for the two-chain combination process. There exists a need for a prorelaxin product recovery process that will provide large
10 enough yields of biologically active relaxin to be commercially feasible. There exists a need for an isolated prorelaxin that can be expressed in a prokaryotic system and subsequently processed to biologically active relaxin not contaminated with host generated materials or other
15 recombinant artifacts that reduce biological activity.

Summary of the Invention

The present invention is based on the unexpected experimental finding that biologically active relaxin in
20 commercially effective amounts and purity can be produced via non-naturally occurring prorelaxin forms. The present invention is based on the unexpected experimental finding that non-naturally occurring prorelaxin forms are successfully folded and processed to biologically active
25 relaxin in recombinant systems in greater yields than realized using naturally occurring prorelaxin.

The present invention is based on the design and construction of nonnaturally occurring prorelaxin forms having a leader sequence, a B-chain, a non-naturally
30 occurring C-peptide, and an A-chain. The leader sequence is comprised of a cleavage site adjacent to the prorelaxin B-chain, and the non-naturally occurring C-peptide is comprised of a cleavage site at the B-chain/C-chain junction and the A-chain/C-chain junction. The present invention is also
35 based on the design of a product recovery process for the production of biologically active relaxin from a non-naturally occurring prorelaxin.

The present invention is accomplished by providing a process for producing relaxin from fermentation of a non-naturally occurring prorelaxin which method comprises:

(a) providing nucleic acid encoding the non-naturally occurring prorelaxin, wherein the prorelaxin comprises a leader sequence, a B-chain, a non-naturally occurring C-chain, and an A-chain, and wherein said leader sequence comprises a first cleavage site adjacent the B-chain sequence and wherein said non-naturally occurring C-chain comprises second and third cleavage sites adjacent the B-chain and the A-chain, respectively; (b) culturing prokaryotic cells containing said nucleic acid encoding said non-naturally occurring prorelaxin, the culturing resulting in expression of said nucleic acid to produce said non-naturally occurring prorelaxin in said prokaryotic cell; (c) isolating and solubilizing said prorelaxin produced by said culture method; (d) refolding said solubilized prorelaxin; (e) excising said leader sequence and said non-naturally occurring C-peptide from said prorelaxin, wherein excision is accomplished through the use of cleaving agents specific for said cleavage sites; and (f) recovering relaxin. The process may further comprise cyclizing the A-chain N-terminal glutamine.

In a preferred embodiment of the present invention, the relaxin is human relaxin of the H2 form.

In one embodiment of the present invention, the excision of the leader sequence and non-naturally occurring C-peptide is through enzymatic cleavage.

In one embodiment of the present invention, the excision of the leader sequence and non-naturally occurring C-peptide is accomplished through the use of trypsin and carboxypeptidase B (CPB), and in another embodiment, Arg C and CPB. The excision of the leader sequence is preferably accomplished through the use of endoproteinases AspN and trypsin. The excision of the non-naturally occurring C-peptide is preferably accomplished through the use of Arg C, trypsin or Lys C with carboxypeptidase B, or with trypsin and Arg C. See Figures 2A-2D.

In one embodiment of the present invention, the solubilized prorelaxin is refolded under conditions of dilute protein concentration. In another embodiment of the present invention, the solubilized prorelaxin may be refolded under
5 controlled oxidation conditions.

In another aspect, the present invention provides an isolated prorelaxin comprising a leader sequence, a B-chain, a non-naturally occurring C-peptide, and an A-chain.

10 **Brief Description of the Drawings**

Figure 1 shows the makeup of DNA encoding a prorelaxin hereof including a non-naturally occurring leader ("ST II") and C-peptide ("mini C") and having cleavage sites after the leader and between the C-peptide and B and A chains as
15 identified.

Figures 2 and 2A to 2D show the leader sequence, the non-naturally occurring C-peptide, and the enzymatic cleavage sites for four constructs of the present invention. (Sequence I.D. nos. 5-8)

20 Figure 3 shows the nucleic acid and amino acid sequence for the plasmid pRB250CTsc. See also Figure 1. (Sequence I.D. no. 9)

Figure 4 illustrates the lineage of the plasmid pRB250CTsc which comprises a gene encoding a non-naturally
25 occurring prorelaxin as depicted in Figure 3.

Figure 5 illustrates the construction of plasmid pRB250C, an intermediate plasmid in the construction of pRB250CTsc.

Figure 6 illustrates the construction of plasmid pRB250,
30 an intermediate plasmid in the construction of pRB250CTsc.

Figure 7 illustrates the construction of plasmid pRB192, an intermediate in the construction of pRB 250.

Figure 8 illustrates the construction of plasmid pRB151, an intermediate in the construction of pRB192.

35 Figure 9 illustrates the construction of plasmid pRB51, an intermediate in the construction of pRB192.

Figure 9A illustrates the Not I-Bam HI fragment from pTR21.

Figure 10 provides a partial sequence of plasmid pRB11.
(Sequence I.D. no. 10)

Figure 10A illustrates the construction of pRB11.

Figure 11 illustrates the construction of plasmid pRB61,
5 an intermediate in the construction of pRB151.

Figure 12 illustrates the construction of plasmid pRA1,
an intermediate in the construction of pRB61.

Figure 12A illustrates the construction of pTF161.

Figure 13 illustrates the construction of pTR591, an
10 intermediate in the construction of plasmid pRA1.

Figure 14 illustrates the construction of pTR561, an
intermediate in the construction of pTR591.

Figure 15 illustrates the construction of pLS33lamB, an
intermediate in the construction of pRB61.

15 Figure 16 provides a partial sequence of pTF271, an
intermediate in the construction of pRB61. (Sequence I.D.
no. 11)

Figure 16A illustrates the construction of pTF271.

Figure 17 illustrates the construction of pRB192C, an
20 intermediate in the construction of pRB250C.

Figure 18 illustrates the construction of pLS32, an
intermediate in the construction of pLS33lamB.

Figure 18A illustrates the sequence of the 291bp Hind
III-Bam HI fragment from pLS8.

25 Figure 19 illustrates the construction of pAPlamB, an
intermediate in the construction of pLS33lamB.

Detailed Description of the Invention

As used herein, "relaxin" is defined as a polypeptide
30 having the amino acid sequence described in Hudson et al.,
(EMBO J. 3, 2333 [1984]) together with naturally occurring
amino acid sequence variants, such as naturally occurring
alleles thereof, which retain the qualitative biological
activity of relaxin.

35 Also falling within the scope of the present invention
are nonnaturally occurring relaxin amino acid substitutions,
insertions or deletions, such as those that can be introduced
using recombinant DNA technology, and covalent or non-

covalent relaxin modifications, for example glycosylation modifications, provided that the final relaxin possesses the qualitative biological activity of naturally occurring relaxin.

5 The term relaxin refers as well to various forms of human and non-human animal relaxin as known to be biologically active in accepted relaxin assays such as the pubic symphysis *in vitro* bioassay [Steinetz et al., Endocrinology 67, 102 (1960)], the rat uterine smooth muscle
10 *in vitro* assay (St. Louis, [J. Can J. Physiol. Pharmacol. 59, 507 (1981)] and measurement of cAMP levels after hormonal stimulus (Braddon, S.A., [Endocrinology 102, 1292 (1978)] and Judson et al., [J. Endocrinology 87, 153 (1980)]).

 Prorelaxin as used herein refers to the precursor of
15 relaxin that comprises the B-, A-, and C-chains, "relaxin" being as defined above. A feature of the prorelaxins hereof is that they contain a non-naturally occurring C-chain, as defined further *infra*.

 The prorelaxin precursor of the present invention is
20 meant to include prorelaxin obtained from a natural source, chemically synthesized, or produced by techniques of recombinant DNA technology. Certain of the non-naturally occurring prorelaxins of the present invention have non-naturally occurring C-chains connecting naturally occurring
25 A- and B-chains. Some of the non-naturally occurring prorelaxins of the present invention have non-naturally occurring C-chains comprised of naturally occurring enzyme cleavage sites, whereas other non-naturally occurring prorelaxins are comprised of non-naturally occurring enzyme
30 cleavage sites.

 The term "C-chain" as used herein refers to the peptide which connects the A and B chains of prorelaxin. A focus of the present invention is the use of "non-naturally occurring C-chain" defined herein as a peptide connecting the A and B
35 chains of prorelaxin that does not occur in the natural protein. The preferred non-naturally occurring C-chain of the present invention is a peptide that connects the A and B chain of prorelaxin and is comprised of amino acids

encoding cleavage sites at the B-/C-chain junction and the A-/C-chain junction. Preferred are C-chains having about 8 to 15 amino acids.

The term prorelaxin "leader", "leader peptide", "leader sequence" or "signal sequence" as used herein refers to the short amino acid sequence that is found at the N-terminus of the prorelaxin hereof. The preferred leader sequence herein is non- or semi-functional in directing prorelaxin to the periplasm of the host prokaryotic cell. Prorelaxin produced by the host cells of the present invention is typically found in and purified from so-called refractile bodies. A particularly preferred leader sequence of the present invention is a truncated STII leader sequence that is used to drive high expression of the prorelaxin, rather than necessarily to achieve secretion of prorelaxin into the periplasm of the host cell. Typically, Lys and Arg are included in the "leader sequence" of the present invention to allow for cleavage of the leader sequence from relaxin.

A typical leader, illustrated as a model herein, is defined as MKKNIAFLLKR. Equivalents thereof would include MKKNIAFLLRK, MKKNIAFLLRR and MKKNIAFLLKK. An attendant feature for a useful leader is that it contain a proteolytic enzyme cleavage site for cleavage from the B-chain.

As used herein the phrase "process for producing relaxin from prorelaxin" or "product recovery process for the production of relaxin" refers to the design and construction of non-naturally occurring prorelaxin as well as the fermentation process for culturing prorelaxin and any subsequent steps for purifying relaxin. Steps for purifying relaxin from prorelaxin expressed in a host culture include but are not limited to, isolating prorelaxin refractile bodies, such as by centrifuging, solubilizing the prorelaxin, refolding the solubilized prorelaxin, cleaving the prorelaxin leader sequence and C-peptide, removing impurities from the relaxin, and providing the relaxin in a form for final formulation. These purification steps are illustrative rather than limiting.

The term "commercially feasible or effective yields or amounts" refers to final relaxin yields derived from the prokaryotic fermentation of a prorelaxin hereof and is defined as being at least about 10 to 100 mg/L, and preferably, greater than about 100 mg/L.

The terms "biological activity", and grammatical equivalents refer to any biological activities exhibited by wild-type human relaxin. The relaxin biological activity may, for example, be determined in accepted relaxin assays such as the pubic symphysis in vitro bioassay [Steinetz et al., Endocrinology 67, 102 (1960)], the rat uterine smooth muscle in vitro assay (St. Louis, [J. Can J. Physiol. Pharmacol. 59, 507 (1981)] and measurement of cAMP levels after hormonal stimulus (Braddon, S.A., [Endocrinology 102, 1292 (1978)] and Judson et al., [J. Endocrinology 87, 153 (1980)]).

The term "cleaving agent" as used herein refers to a reagent used to cleave the prorelaxin hereof specifically so as to release or excise certain components, such as the leader sequence or the C-peptide, as desired. Suitable cleaving agents herein include enzymes, such as endoproteases, e.g., endoproteinase Lys C, endoproteinase Arg C, endoproteinase Asp N; trypsin; carboxypeptidase B; prohormone convertase (PC), e.g., furin, PC1, PC2, KEX2; subtilisin, or its mutants; and chemical agents, such as organic or inorganic acids, hydroxylamine, N-bromosuccinimide, and cyanogen bromide.

Hydrolysis of peptide bonds catalyzed by a variety of proteolytic enzymes is taught in The Enzymes, 3rd ed., Boyer, Ed., Academic Press, Vol. III, [1971]; Meth. Enzymol. Vol. XIX, Perlman and Lorand, Ed. New York: Academic Press [1970]; Enzymol. Vol. XLV, Lorand, Ed. New York: Academic Press [1976]; Drapeau, [J. Biol.Chem. 253: 5899 (1978)] and Drapeau, [Meth. Enzymol. 47, 89 (1977)]. For an extensive listing of chemical agents, see Witcop in Advances in Protein Chemistry, Anfinsen et al., ed., Vol 16 pg. 221-321, Academic Press, New York [1961], including Table III on p. 226.

Other cleavage agents suitable herein are deemed to be understood by those skilled in the art keeping in mind the desired junction for cleavage and whether the reagent can act on reduced or oxidized forms of prorelaxin. Conditions used
5 for cleavage of the non-naturally occurring prorelaxin will depend upon the cleaving agent employed, and the conditions will be readily apparent to one skilled in the art given the cleavage agent employed.

In the present invention, the non-naturally occurring
10 prorelaxin is designed and constructed to comprise the codon(s) necessary to achieve cleavage by the desired cleaving agent at desired position or positions, i.e., after a leader sequence or to excise a C-peptide. It may be necessary to insert the appropriate codons either upstream
15 and preferably adjacent to the 5'-terminal codon of the sequence encoding the desired polypeptide component, in this case relaxin B-chain or A-chain, or downstream, and preferably adjacent to the carboxy terminal codon of the desired component of the polypeptide, or both if the desired
20 component to be isolated is an internal amino acid sequence of the expected translation product.

In the present invention, it is efficient that the prorelaxin leader sequence and C-peptide are excised by the same cleavage method. Any enzyme or chemical that can cleave
25 the cleavage sites available at the leader sequence/B-chain junction, the B-/C-chain junction and the A-/C-chain junction can be used as long as the desired hormone, relaxin, can be generated.

The oligonucleotides are readily synthesized using
30 techniques well known in the art such as that described by Crea et al., Proc. Nat'l. Acad. Sci. USA 75:5765 (1978), or Kunkel et al., Methods in Enzymol. 154 367 (1987).

Mutants with more than one amino acid substituted may be generated in one of several ways. If the amino acids are
35 located close together in the polypeptide chain, they may be mutated simultaneously using one oligonucleotide that codes for all of the desired amino acid substitutions. If however, the amino acids are located some distance from each other

(separated by more than ten amino acids, for example) it is more difficult to generate a single oligonucleotide that encodes all of the desired changes. Instead, one of two alternative methods may be employed. In the first method, a separate oligonucleotide is generated for each amino acid to be substituted. The oligonucleotides are then annealed to the single-stranded template DNA simultaneously, and the second strand of DNA that is synthesized from the template will encode all of the desired amino acid substitutions. The alternative method involves two or more rounds of mutagenesis to produce the desired mutant.

Another method for making mutations in the nucleic acid sequence encoding wild-type prorelaxin or a variant molecule known in the art, involves cleaving the nucleic acid sequence encoding the starting prorelaxin molecule at the appropriate position by digestion with restriction enzymes, recovering the properly cleaved nucleic acid, synthesizing an oligonucleotide encoding the desired amino acid sequence and flanking regions such as polylinkers with blunt ends (or, instead of polylinkers, digesting the synthetic oligonucleotide with the restriction enzymes also used to cleave the prorelaxin encoding nucleic acid, thereby creating cohesive termini), and ligating the synthetic nucleic acid into the remainder of the prorelaxin encoding structural gene.

PCR mutagenesis is also suitable for making the prorelaxin variants of the present invention, for example, as described in U.S. Patent No. 4,683,195, issued 28 July 1987, and in Current Protocols in Molecular Biology, Ausubel et al., eds. Greene Publishing Associates and Wiley-Interscience, Volume 2, Chapter 15, 1991. While the following discussion refers to DNA, it is understood that the technique also finds application with RNA. Mutations at separate positions can be introduced simultaneously by either using a mutant second primer or performing a second PCR with different mutant primers and ligating the two resulting PCR fragments simultaneously to the vector fragment in a three (or more)-part ligation.

The relaxin nucleic acid derived from RNA, cDNA, genomic DNA, synthetic DNA or a combination of DNA is inserted into a replicable vector for further cloning (amplification of the nucleic acid) or for expression. Construction of suitable
5 vectors containing the desired coding and control sequences employs standard recombinant techniques. Isolated plasmids or nucleic acid fragments are cleaved, tailored, and religated to form the desired plasmid.

Many vectors are available, and selection of the
10 appropriate vector will depend on 1) whether it is to be used for nucleic acid amplification or for nucleic acid expression, 2) the size of the nucleic acid to be inserted into the vector, and 3) the host cell to be transformed with the vector. Each vector contains various components
15 depending on its function (amplification of nucleic acid or expression of nucleic acid) and the host cell for which it is compatible. The vector components generally include, but are not limited to, one or more of the following: a signal sequence, an origin of replication, one or more marker genes,
20 an enhancer element, a promoter, and a transcription termination sequence.

The preferred replicable vector of the present invention is one containing a leader sequence that allows for expression of the non-naturally occurring prorelaxin and
25 correct N-terminal processing of the prorelaxin B-chain, the tryptophan (trp) promoter, the λ_{10} termination sequence, a PBR322 origin of replication, an antibiotic resistance gene and the relaxin A and B chains connected by a non-naturally occurring C-peptide wherein said C-peptide is comprised of
30 enzymatic cleavage sites at the B-/C- chain and A/C-chain junction.

Prokaryotes are the preferred host cells for the initial cloning steps of prorelaxin. They are particularly useful for rapid production of large amounts of nucleic acid, for
35 production of single-stranded nucleic acid templates used for site-directed mutagenesis, for screening many mutants simultaneously, and for nucleic acid sequencing of the mutants generated. Examples of prokaryotes, e.g. E.coli, and

expression vectors, suitable for use in producing prorelaxin are, for example, those disclosed in WO 90/02798 (published 22 March 1990). Prokaryotes used for cloning of prorelaxin DNA sequences also include for example, E.coli K12 strain 294
5 (ATCC No. 31446), E.coli B, and E.coli X1776 (ATCC No. 31537).

Prokaryotes also are used for expression. Suitable host cells for cloning or expressing the vectors herein are the E.coli cells. E.coli strain W3110 (F⁻, 1⁻, prototrophic,
10 ATCC No. 27325) is a particularly preferred parent host because it is a common host strain for recombinant DNA product fermentations. Preferably, the host cell should secrete minimal amounts of proteolytic enzymes. Strain W3110 may be modified to effect a genetic mutation in the genes
15 encoding proteins, with examples of such hosts including E.coli W3110 strain 1A2, which has the complete genotype tonAA; E.coli W3110 strain 9E4, which has the complete genotype tonAA ptr3; E.coli W3110 strain 27C7 (ATCC 55,244), which has the complete genotype tonAA ptr3 phoAAE15 Δ (argF-lac)169 ompTA degP41kan^r;
20 E.coli W3110 strain 37D6, which has the complete genotype TonAA ptr3 phoAAE15 4(argF-lac)169 ompTA degP41kan^rrbs7 Δ ilvG; E.coli strain W3110 strain 40B4m which is strain 37D6 with a non-kanamycin resistant degP deletion mutation; and an E.coli strain having mutant
25 periplasmic protease disclosed in U.S. Pat. No. 4,946,783, issued 7 August 1990.

Cloning and expression methodologies are well known in the art and are, for example, disclosed in the foregoing published PCT patent application (WO90/02798).

30 Fermentation of the prorelaxin is carried out through methodologies well known in the art and are, for example, disclosed in Elander (Genetic Engineering Technology in Industrial Pharmacy edited by John M. Tabor, published by Marcel Dekker, Inc. pg. 115-129 [1989]), however many
35 fermentation variables exist which remain to be optimized for each fermentation process. The major goals of fermentation development are to optimize cell mass and maximize product accumulation. Large scale fermentation refers to

bodies from the fermentation broth, the prorelaxin refractile bodies are solubilized and refolded.

Solvents used to solubilize the protein in refractile bodies include, but are not limited to, Guanidine-HCl (GuHCl) (up to 8M), Urea (up to 8M), SDS, Alkaline pH (>8.0), acid pH (<3.0) and Acetonitrile/propanol. The preferred solubilization buffer for the prorelaxin refractile bodies of the present invention is GuHCl, 3.5-4.0 M or Urea, 2-8M. In the present invention, PEI (polyethyleneimine) is used to retain prorelaxin in a soluble form while precipitating contaminants.

Refolding of solubilized protein can be accomplished by lowering or removing the solubilizing agent (e.g., by dialysis or dilution) with oxidation of reduced protein occurring prior to or concomitant with refolding for proteins containing disulfide bridges. In the present invention, it is preferred that the folding step take place in an oxidative environment using a redoxo buffer. In the present invention it is preferred that refolding be carried out at as dilute a concentration as feasible, taking into consideration workable volumes of solutions and possible loss due to high dilution for subsequent purification steps. It is most preferred to use dilutions in the range of 60-100 times refractile body weight in grams. During the solubilization and refolding process, it is also preferable to minimize exposure to conditions which result in derivatization of prorelaxin amino acid side chains (e.g., prolonged exposure to pH values of greater than 9.0). For refolding proteins, such as prorelaxin, that contain cysteine residues and in the naturally occurring form contain disulfide bonds, the reduction/oxidation conditions present in the solubilization and refolding steps are critical and protein specific. In the present invention it is preferred that the steps of solubilization and refolding take place at 2-8°C.

After refolding, purification processes are necessary to remove other proteins, contaminating nucleic acids present in the inclusion body, and folding intermediates and to isolate and concentrate the prorelaxin. The following

fermentation in a fermentor that is at least approximately 1000 liters in volumetric capacity, i.e., working volume, leaving adequate room for headspace. Small scale fermentation refers generally to fermentation in a fermentor that is no more than approximately 100 liters in volumetric capacity, preferably no more than approximately 10 liters.

Isolation of crude product from the fermentation broth can be accomplished by the use of filtration, centrifugation, and/or settling, sedimentation and decanting or a combination of techniques. Isolation of crude product in the form of refractile bodies requires a first step of cell disruption in order to release the refractile body from the cell. Methods of cell disruption include sonication, passage through homogenizers, and cell lysis accomplished through the use of lysozyme, detergent or other agents.

Once the refractile bodies are released from the cell, the bodies may be separated from the remaining fermentation solution based on differences in physical and chemical properties such as size and solubility. Sedimentation refers to settling in a simple gravitational field, whereas centrifugation requires production of enhanced settling velocities by centrifugal forces. In the present invention, the preferred manner of isolating crude prorelaxin from the fermentation broth includes a form of mechanical cell disruption, to release the prorelaxin refractile body from the cell, followed by any centrifugal technique which allows for separation of refractile bodies from light solid wastes and liquids. In the present invention, the preferred form of mechanical cell disruption is by homogenization while the preferred centrifugal technique is a high volume, continuous flow, solid bowl centrifugation.

In the case where proteins are expressed in the form of intracellular refractile bodies, the product recovery process will include process steps for solubilizing the refractile bodies, renaturing the solubilized protein and where appropriate, a controlled oxidation step to obtain useful product. After isolation of crude prorelaxin refractile

examples are exemplary of suitable purification procedures: fractionation on immunoaffinity or ion-exchange columns; ethanol precipitation; reverse phase HPLC; chromatography on silica columns; electrophoresis; ammonium sulfate precipitation; gel filtration; ultrafiltration/diafiltration; metal chelate chromatography; and hydrophobic interaction chromatography. Chromatographic matrices are commercially available for use in purification of desired product from contaminants; a review of chromatographic matrices is given in Marston et al., Supra and Section VII from [Guide to Protein Purification] edited by Deutscher, published by Academic Press, Inc. 309 (1990)].

In the present invention, it is preferred that the cleavage of prorelaxin to relaxin take place after prorelaxin refolding and subsequent removal of solubilizing agents. Methods of cleavage include chemical cleavage and enzymatic cleavage as discussed herein.

In the present invention, the preferred process includes a process step for cyclization of the relaxin A-chain N-terminal glutamine. Any process for cyclization of the A-chain N-terminal glutamine can be used. Examples of such a process are heat treatment, preferably under slightly acidic conditions, and treatment with nucleophilic reagents, such as imidazole at neutral pH. Any process steps used to cyclize the N-terminal A chain may require additional purification steps to remove any contaminants caused by the cyclization procedure itself, and may include centrifugation, column chromatography or precipitation techniques.

Final product purification may include steps of chromatography, organic solvent removal, ultrafiltration/diafiltration which provide relaxin in a form for final formulation.

Typically, the relaxin used in the method of this invention is formulated by mixing it at ambient temperature at the appropriate pH, and at the desired degree of purity, with pharmaceutically acceptable carriers, i.e., carriers that are non-toxic to recipients at the dosages and concentrations employed. Suitable carriers and their

formulations are described in Remington's Pharmaceutical Sciences, 16th ed., 1980, Mack Publishing Co., edited by Oslo et al. These compositions will typically contain an effective amount of the relaxin, for example, from on the
5 order of about 0.0003 upwards of about 8 or more mg/ml, together with a suitable amount of carrier to prepare pharmaceutically acceptable compositions suitable for effective administration to the patient.

The pH of the formulation preferably ranges anywhere
10 from about 3 to about 8. Formulation in an acetate buffer at pH 5 is a suitable embodiment. The preferred formulation for relaxin is a buffered or unbuffered solution, and is preferably 20mM sodium acetate, pH 5.0.

Compositions particularly well suited for the clinical
15 administration of relaxin include sterile aqueous solutions or sterile hydratable powders such as lyophilized protein. Typically, an appropriate amount of a pharmaceutically acceptable salt is also used in the formulation to render the formulation isotonic.

20 Sterility is readily accomplished by sterile filtration through (0.2 micron) membranes. Relaxin ordinarily will be stored as an aqueous solution, although lyophilized formulations for reconstitution are acceptable.

The relaxin composition will be formulated, dosed, and
25 administered in a fashion consistent with good medical practice. Factors for consideration in this context include the particular disorder being treated, the particular mammal being treated, the clinical condition of the individual patient, the cause of the disorder, the site of delivery of
30 the agent, the method of administration, the scheduling of administration, and other factors known to medical practitioners. The "therapeutically effective amount" of relaxin to be administered will be governed by such considerations, and is the minimum amount necessary to
35 prevent, ameliorate, or treat the disorder.

As a general proposition, the pharmaceutically effective amount of the relaxin administered per dose will be in the range of about 0.001 to 100 mg/kg of patient body weight per

day with the typical range of relaxin used being 0.005 to 50 mg/kg/day.

The invention will be more fully understood by reference to the following examples. They should not, however, be construed as limiting the scope of the invention. All literature citations are expressly incorporated by reference.

Example 1

Construction of Expression Vehicle for a Model of Non-Naturally Occurring Prorelaxin

The plasmid pRB250CTsc is comprised of prorelaxin (the relaxin A and B chains derived from the sequence disclosed in Hudson et al., [EMBO J. 3, 2333 (1984)] having a non-naturally occurring leader sequence and a non-naturally occurring C-peptide containing trypsin and trypsin/carboxypeptidase enzymatic cleavage sites at the A/C chain junction and the B/C chain junction, as is shown in Figures 1 and 3. The transcriptional and translational sequences required for expression of the prorelaxin gene in *E. coli* are provided by the tryptophan (trp) promoter derived from pHCH207-1 (deBoer et al., from [Promoters: Structure and Function, eds. Rodriguez and Chamberlain, publisher M.J. Praeger, New York 462 (1982)]). The lambda t₀ transcriptional terminator [Scholtisses, et al., NAR 15, 3185 (1987)] is situated adjacent to the prorelaxin termination codon. Plasmid pRB250CTsc confers tetracycline resistance upon the transformed host. Plasmid pRB250CTsc has an origin of replication from a pBR322 vector [Sutcliffe, Cold Spring Harbor symposium on Quantitative Biology 43, 77 (1978)].

Plasmid pRB250CTsc also has 9 amino acids from the *E. coli* heat-stable enterotoxin II (STII) gene [Picken et al., Infect. Immun. 42, 269 (1983)] followed 3' by amino acids Lys and Arg.

The STII 9 amino acids plus Lys and Arg are located 3' to the Trp promoter and allow for high level expression of the non-naturally occurring prorelaxin having a convenient cleavage site provided, thereby allowing for generation of the correct N-terminal processing of the prorelaxin B-chain

through enzymatic cleavage. The STII 9 amino acids do not encode a functional leader sequence.

Plasmid pRB250CTsc: The plasmid pRB250CTsc was constructed in several steps, as shown in Figure 4, using as
 5 intermediate plasmids pRB250, containing the trp promoter, pRB250C, containing the non-naturally occurring prorelaxin coding sequences on a 250 base pair Xba I to Hind III fragment, and pdh108, containing the lambda t_o transcriptional terminator on a 412 base pair Stu I to Bam
 10 HI fragment.

Plasmid pRB250: The plasmid pRB250 results in the trp promoter/operator being ligated to 9 amino acids of the heat stable enterotoxin II (ST II) leader sequence (MKKNIAFLL) described in Picken et al., [*Infect. Immun.* 42, 269 (1983)]
 15 plus codons for Lys and Arg. pRB250 was prepared by ligating together three DNA fragments as shown in Figure 7. The first of these was the vector pRB192, shown in Figure 7, from which the small fragment from BssHII to XbaI had been removed. The second fragment is a synthetic duplex, Rel 60, encoding the
 20 STII 9 amino acids plus Lys and Arg:

MetLysLysAsnIleAlaPheLeuLeuLysArg

5'-CTAGAATTATGAAAAAGAAATATCGCATTTCTTCTTAAACGGG-3'

3'-TTAATACTTTTTCTTATAGCGTAAAGAAGAATTTGCCCTGA-5'

The third fragment is the HinfI to BssHII fragment from
 25 plasmid pRB51, the construction of which is described in Figure 9.

Plasmid pRB250C: The plasmid pRB250C results in the Trp promoter being ligated to the STII leader 9 amino acids plus amino acids Lys and Arg and also contains the non-naturally
 30 occurring prorelaxin as well as a tetracycline resistance gene minus the naturally occurring tetracycline resistance gene promoter.

pRB250C was prepared by ligating together three fragments as shown in Figure 6. The first of these was the
 35 vector pRB192 from which the small fragment from Xba I to Bam HI had been removed. The second fragment was a Not I to Bam HI fragment from pRB192C which comprised the C-peptide from the non-naturally occurring prorelaxin. The third fragment

was a Not I to Xba I approximately 75 base pair fragment from pRB250.

Plasmid pdh108: Plasmid pdh108 contains the lambda transcription terminator as described in Scholtissek et al.,
5 *Supra*.

Plasmid pRB192: The plasmid pRB192 contains the Trp promoter ligated to amino acid methionine followed by 9 amino acids of porcine growth hormone plus Lys and Arg and also contains naturally occurring prorelaxin. pRB192 was prepared
10 by ligating together three fragments as shown in Figure 7. The first of these fragments was the vector pRB151 from which the small fragment Xba I to Bss HII had been removed. The second fragment was the synthetic duplex, Rel 52, having the sequence:

15 MetPheProAlaMetProLeuSerSerLysArg

5'-CTAGAATTATGTTCCCAGCTATGCCTCTATCTAGTAAACGGG-3'

3'-TTAATACAAGGGTCGATACGGAGATAGATCATTTGCCCTGA-5'

which is methionine plus 9 amino acids of porcine growth hormone plus Lys and Arg. The third fragment was a Hinf I to
20 Bss HII 46 base pair fragment from the vector pRB51.

Plasmid pRB192C: Plasmid pRB192C, results in the Trp promoter being ligated to methionine followed by 9 amino acids of porcine growth hormone plus Lys and Arg and further contains non-naturally occurring prorelaxin. Plasmid pRB192C
25 contains a promoterless tetracycline resistance gene. pRB192C was prepared by ligating together three fragments as shown in Figure 18. The first fragment was the large vector fragment from BssHII to EcoRV from the plasmid pRB192. The second fragment was a synthetic fragment, Rel 58, containing
30 the non-naturally occurring prorelaxin of the sequence:

GlnIleAlaIleCysGlyMetSerThrTrpSerLysArgLysProThrGlyTyr

5'-CGCGCAGATTGCCATTTGCGGCATGAGCACCTGGAGCAAAGGAAACCCACTGGTTAT

3'-GTCTAACGGTAAACGCCGTACTCGTGGACCTCGTTTTTCCTTTGGGTGACCAATA

GlySer

35 GGTTCT-3'

CCAAGAGC-5'

The third fragment was the *Taq* I to *Eco* RV 200 base pair fragment of pRB192 containing the relaxin A chain and the 5' end of the tetracycline resistance gene.

Plasmid pRB151: Plasmid pRB151 results in the Trp promoter being ligated to the preprorelaxin coding sequence and was prepared by ligating together two fragments as shown in Figure 8. The first of these is a *Pst* I to *Xba* I vector fragment from plasmid pRB61. The second fragment contains the Trp promoter on a *Pst* I to *Xba* I fragment from the plasmid pHG207-1 as described in (deBoer et al., from [Promoters: Structure and Function, eds. Rodriguez and Chamberlain, publisher M.J. Praeger, New York 462 (1982)]).

Plasmid pRB51: Plasmid pRB51 results in the alkaline phosphatase promoter being ligated to the full STII leader sequence and also contains the naturally occurring prorelaxin sequence. pRB51 was prepared by ligating together two fragments as shown in Figure 9. The first fragment was the large vector fragment from *Not* I to *Bam* HI from plasmid pRB11; the second fragment was the small *Not* I to *Bam* HI fragment from pTR21 encoding prorelaxin amino acids 12 to 161 and whose sequence is shown in Figure 9A. pRB11 is an expression plasmid designed to express the relaxin B-chain in *E.coli* with the aid of the STII signal sequence. The transcriptional and translational sequences required for expression are provided by the AP promoter, and the tryptophan (*trp*) and STII Shine-Dalgarno sequences. The plasmid origin and tetracycline resistance gene of pRB11 were derived from an altered pBR322 plasmid in which the nucleotide sequence between the *Ava*I and *Pvu*II endonuclease restriction sites has been deleted, thereby resulting in a higher plasmid copy number per cell. The coding sequence for relaxin B-chain was obtained from a preprorelaxin H2 cDNA clone [Hudson et al., *EMBO J.* 3, 2333 (1984)]. The alkaline phosphatase (AP) promoter and the heatstable enterotoxin II (STII) signal sequence is described in Chang et al., *Gene* 55, 189 [1987]. The sequence of pRB11 is shown in Figure 10.

Plasmid pRB61: Plasmid pRB61 results in the alkaline phosphatase promoter being ligated to the naturally occurring

preprorelaxin coding sequence. pRB61 was prepared by ligating together four fragments as shown in Figure 11. The first fragment was an Eco RI to Hind III vector fragment from vector pLS33lamB as shown in Figure 15. The second fragment
 5 was an Eco RI to Xba I, 412 base pair fragment from plasmid pTF271 as shown in Figure 16. The plasmid pTF271 is designed to express the first 243 amino acids of mature human tissue factor into the periplasm of E.coli with the aid of the STII signal sequence. The transcriptional and translational
 10 sequences required for expression are provided by the alkaline phosphatase promoter and the trp and STII Shine-Dalgarno sequences. The coding sequence for human tissue factor is described by Fisher et al., Throm Res 48 89 (1987). The alkaline phosphatase promoter, tryptophan (trp) and heat
 15 stable enterotoxin II (STII) Shine-Dalgarno sequences, and the STII signal sequence were derived from pHGH-1 [Chang et al., Gene 55 189 (1987)]. The plasmid origin and tetracycline resistance gene were derived from pBR322 [Sutcliffe Cold Spring Harbor Symposia on Quantitative
 20 Biology Vol. 43 77 (1978)].

The third fragment is the small Xba I to Bgl II fragment from plasmid pPreProRelH2Trp207. pPreProRelH2Trp207 is a derivative of pHGH207 (as described in U.S. Patent 4,663,283 issued 5 May 1987) in which the HGH coding sequence has been
 25 replaced with that of preprorelaxin. The fourth fragment is a 24 base pair Bgl II to Hind III fragment from the vector pRA1.

Plasmid pLS33lamB: pLS33lamB was constructed as shown in Fig. 15 by ligating together three DNA fragments. The
 30 first of these was the vector pLS32, described below, in which the small XbaI-BstEII fragment had been removed. The second was a 75-bp XbaI-EaeI fragment from pAPlamB, described below, encoding the lamB signal sequence. The third was a 46-bp synthetic DNA duplex with the following sequence:
 35 5'-GGCCACTCTGTGCGGTGCTGAACTGGTTGACGCTCTGCAGTTTGTTCG-3'

3'-TGAGACACGCCACGACTTGACCAACTGCGAGACGTCAAACAAACGCCACTG-5'

The above sequence encodes amino acids 4-18 of mature IGF-I.

pLS32 results in the fusion of the IGF-I coding sequence to that of the heat-stable enterotoxin II (STII) signal sequence and was prepared by ligating together four DNA fragments, as shown in Figure 18. The first of these was the
 5 vector pTF2A12 [Paborsky et al., Biochemistry 28, 8072 (1989)] from which the small NsiI-BamHI fragment containing the tissue factor gene had been removed. The STII signal sequence is described by Picken et al., Infect. Immun. 42, 269 1983]. The second fragment was a 55-bp synthetic duplex
 10 encoding the first 18 amino acids of mature IGF-I. This duplex has the following sequence:

5'-GGTCCCGAAACTCTGTGCGGTGCTGAAGTGGTTGACGCTCTGCA

3'-ACGTCCAGGGCTTTGAGACACGCCACGACTTGACCAACTGCGAGACGT

GTTTGTTTGCG-3'

15 CAAACAAACGCCACTG-5'

The third piece in the ligation was a 154 bp BstEII to HindIII fragment from pK1ZZ IGF-I encoding the remaining amino acids 19-70 of IGF-I. pK1ZZIGF-I is a kanamycin-resistant plasmid containing a lac promoter attached to a
 20 Protein A promoter attached to a Protein A signal, attached to two consensus z regions from Protein A that bind IgGs and secrete proteins, fused using two codons encoding an Asn-Gly interface to a synthetic IGF-I gene and also containing an F region to five high copy number. This plasmid is similar
 25 to pZZ-IGF-I described in EP publication no. 230,869 published 5 August 1987, where ampicillin gene is replaced by a kanamycin gene. The last fragment shown in Figure 18B in the construction of pLS32 was a 291-bp HindIII-BamHI fragment from the plasmid pLS8. This last fragment is simply
 30 the coding sequence for the start of the tetracycline gene of pBR322 [Sutcliffe, Cold Spring Harbor Symposia on Quantitative Biology 43 77 (1978)] in which a HindIII restriction site was engineered immediately upstream of the methionine start codon.

35 Plasmid pAPlamB: The plasmid pAPlamB was constructed as shown in Figure 19, by ligating together two DNA fragments, and results in the placement of the lamB signal coding sequence downstream of the AP promoter and the trp

Shine-Dalgarno sequence. Included in the ligation was the vector pRA1 in which the small XbaI-BglII fragment had been removed. This plasmid is a derivative of pGH1 [Chang et al., Gene 55, 189 (1987)], which latter plasmid contains the AP promoter, the STII signal and DNA encoding HGH. pRA1 differs from pGH1 in that it contains DNA encoding relaxin A chain (the sequence of which is described in U.S. Pat. No. 4,758,516) rather than hGH and it contains a convenient BglII restriction site downstream of the promoter and ribosome binding site. The second piece in the ligation was a 80-bp synthetic DNA duplex with the following sequence, which encodes the lamB signal sequence, which has been described by Clement and Hofnung, Cell 27, 507 [1981]:

5'-CTAGAATTATGATGATTACTCTGCGCAAACCTTCCTCTGGCGGTTG
 3'-TTAATACTACTAATGAGACGCGTTTGAAGGAGACCGCCAAC
 CCGTCGCAGCGGGCGTAATGTCTGCTCAGGCCATGGCCA-3'
 GGCAGCGTCGCCCCGATTACAGACGAGTCCGGTACCGGTCTAG-5'

Plasmid pRA1: Plasmid pRA1 results in the alkaline phosphatase promoter being fused to the STII leader sequence and further contains the coding sequence of the Relaxin A chain. pRA1 was prepared by ligating together three fragments as shown in Figure 12. The first was a Nsi I to Nhe I large vector fragment from the plasmid pTF161, a derivative of pGH1 [Chang et al. Gene 55, 189 (1987)] and pTF111 [Paborsky et al., Biochemistry 28, 807 (1989)] as described in Figure 12A. The second fragment is the synthetic duplex, Rel 36, having the sequence:

5'-CAACTCTACAGTGCATTGGCTAATAAATGTTGCCATGTTGGTTGTACC
 3'-ACGTGTTGAGATGTCACGTAACCGATTATTTACAACGGTACAACCAACATGG
 AAAA-3'
 TTTTCTAG-5'

The third fragment was a 224 base pair Bgl II to Nhe I fragment from plasmid pTR591.

Plasmid pTR591: Plasmid pTR591 resulted in the trp promoter ligated to human prorelaxin. pTR591 was prepared by ligating together three fragments as shown in Figure 13. The first fragment was the large Bgl II to Bam HI vector fragment from the plasmid pTR561 as shown in Figure 14 and

described in WO 90/13659. The second fragment was the 26 base pair Alu I to Bgl II fragment from pTR561, while the third fragment was a 344 base pair Alu I to Bam HI fragment from PBR322.

- 5 In addition to the plasmid pRB250CTsc, other plasmids comprised of prorelaxin having non-naturally occurring leader and C-peptides were constructed as shown in Figure 2.

Examples 2 to 4

- 10 The prorelaxin expression vectors, pRELClIII, pRELCAspN, and pRELCLysC, comprised of non-naturally occurring leader-C-peptides having enzymatic cleavage sites (See Figure 2) are constructed as above for pRB250CTsc with appropriate substitution of synthetic C-peptide coding sequences.

15

Example 5

A process for producing relaxin from a non-naturally occurring prorelaxin using trypsin and CPB.

- 20 **Fermentation and initial isolation of the refractile bodies:**

W3110tonA transfected with pRB250CTsc is used in fermentation. An LB shake flask grown at 37°C for about 8 hours is used to inoculate at 60 L seed fermentation. The 60 L culture is grown at 37°C to an OD550 of 45 +/- 5 (approximately 8-9 hours), then used to inoculate a 1000 L production fermentation. The 1000 L culture is grown at 37°C and harvested 8 hours after the addition of Indoleacetic Acetic Acid (IAA), usually 12-16 hours after the time of inoculation. The medium employed is LB Flask - Luria broth + 5 micrograms tetracycline/mL.

30 The harvested cells are then treated with the process steps outlined below.

At harvest time the cells are killed by passing the broth through a heat-kill apparatus. The mixture is cooled to 2-8°C, EDTA is added to a final concentration of 5mM and the pH is adjusted to 5.5. The cells are broken, (e.g., in Gaulin homogenizer) and the refractile bodies are collected

by centrifugation (eg Alfa Laval AX213). The pelleted refractile bodies are frozen and stored at about -70C.

Extraction, folding and purification of mini-C prorelaxin:

5 The frozen refractile bodies are solubilized in 14L to 20L of extraction buffer (3.5M guanidine hydrochloride/50mM Tris/0.2% EDTA, pH 8.5) per kg of refractile bodies.

The prorelaxin is refolded by diluting the above extract with 50mM Tris/0.2% EDTA, pH 8.5 to a final volume of 60L per 10 kg of refractile bodies. Cystamine (0.113g/L) and cysteine (0.606g/L) are added and the mixture stirred for about an hour to allow folding of the prorelaxin. After completion of the refolding polyethyleneimine is added to a final concentration of about 0.05 to 0.1%. The resulting 15 suspension is stirred gently for about an hour. After an additional dilution to 120L/kg with 50mM Tris/0.2% EDTA, pH 8.5 the suspension is stirred gently for an additional hour.

The solids are removed by centrifugation (e.g. CEPA Z101 or Alfa Laval AX 213) and the resulting supernatant is 20 filtered through a depth filter (e.g. CUNO). The clear solution is loaded onto a silica column equilibrated in 3.5M urea/50mM Tris/0.2% EDTA, pH 8.5. After the column is washed with 5M urea/50mM Tris/0.2% EDTA, pH 8.5, the folded prorelaxin is eluted with 5M urea/50mM Tris/0.2% EDTA/0.5M 25 TMAMC, pH 8.5.

The resulting pool is further purified by cation exchange chromatography (e.g. S-Sepharose Fast Flow). The solution is directly applied to the column equilibrated in 3.5M Urea/50mM Tris/0.2% EDTA, pH 8.5. The column is washed 30 with the same buffer. The prorelaxin is eluted with the same buffer containing 0.5M NaCl.

Enzymatic cleavage of mini-C prorelaxin:

The pool from the ion exchange column is concentrated 35 to about 5-10mg/mL and diafiltered into 50mM Tris/5mM CaCl₂ pH 8.5 on a 5K cutoff membrane (e.g. Filtron PES Omega).

Trypsin is added to the solution at a 1:100 w/w ratio (eg 1 MG trypsin per 100 mg of prorelaxin). After 30 minutes

Carboxypeptidase B is added to the mixture (0.2rEU of CPB per mg of prorelaxin). The progress of the cleavage reaction is followed by analytical reversed phase chromatography on a C4 or C18 column. After completion the reaction is stopped by the addition of glacial acetic acid (30mL per liter of reaction mixture).

Cyclization of the N-terminal glutamine of the A-chain:

The acidified solution is heated to about 85C, held there for about an hour and then cooled down to about 10C. The resulting suspension is diluted by the addition of an equal volume of 0.5M acetic acid and centrifuged. The pellet can be washed with 0.5M acetic acid and recentrifuged. The resulting supernatants are combined and filtered.

15

Purification of relaxin:

The clear supernatants are loaded onto a cation exchange column (eg S-Sepharose high performance) equilibrated in 0.5M acetic acid/50mM Tris/5mM CaCl₂, pH 3.5. After completion of the loading the column is first washed with the equilibration buffer, then with 50mM MES, pH 7.0 and 50mM MES/125mM NaCl, pH 7.0. The relaxin is eluted with a gradient of NaCl from 125mM to 140mM in 50mM MES at pH 7.0.

Reversed phase chromatography is performed on a C4 or C18 silica column equilibrated in 0.1% phosphoric acid. Relaxin is eluted with a gradient of the equilibration buffer and 0.1% phosphoric acid/80% acetonitrile.

The resulting pool is directly loaded onto a high performance cation exchange column (eg MONO S) equilibrated in 20mM MES/5% ethanol, pH 6.0. Relaxin is eluted with a gradient of NaCl from 10mM to 32mM in 20mM MES/5% ethanol, pH 6.0.

The relaxin is then formulated by size exclusion chromatography (e.g., Sephadex G-15) or by ultra- and difiltration on a 5K cutoff membrane (e.g., Amicon YM-5, Filtron Omega) with either 10mM citrate/isotonic saline, pH 5.0 or 20mM sodium acetate, pH 5.0.

Concluding Remarks:

The foregoing description details specific methods that can be employed to practice the present invention. Having detailed specific methods initially used to characterize, 5 prepare and use the particular compounds hereof, and further disclosure as to the specific model systems employed, those skilled in the art will well enough know how to devise alternative reliable methods for arriving at the same information and for extending this information to other 10 compounds and systems. Thus, however detailed the foregoing may appear in text, it should not be construed as limiting the overall scope hereof; rather, the ambit of the present invention is to be governed only by the lawful construction of the appended claims.

WHAT IS CLAIMED IS:

1. A process for producing relaxin from a non-naturally occurring prorelaxin wherein said prorelaxin comprises a leader peptide, the B-chain, a non-naturally occurring C-chain, and the A-chain, and wherein said leader peptide comprises a cleavage site adjacent the B-chain and wherein said non-naturally occurring C-chain comprises cleavage sites adjacent the B-chain and the A-chain, which method comprises removing the said leader and non-naturally occurring C-peptide from said prorelaxin using a cleaving agent at said cleavage sites and recovering relaxin.
2. The process of Claim 1 wherein the relaxin is H2 human relaxin.
3. The process of Claim 1 wherein said cleaving agent is one or more enzymes.
4. The process according to Claim 3 wherein said enzymes are selected from the group consisting of endoproteinase Asp N, trypsin, endoproteinase Lys C, endoproteinase Arg C and carboxypeptidase B.
5. The process according to Claim 4 wherein said enzymes are trypsin or endoproteinase Arg C in combination with carboxypeptidase B.
6. The process according to Claim 4 wherein said enzymes are Lys C in combination with carboxypeptidase B.
7. The process according to Claim 4 wherein said enzymes are Asp N in combination with Lys C.
8. The process according to Claim 1 wherein said prorelaxin is produced recombinantly by providing DNA encoding it within an operative expression vector

transfected into host cells, culturing said transfected cells and isolating said prorelaxin.

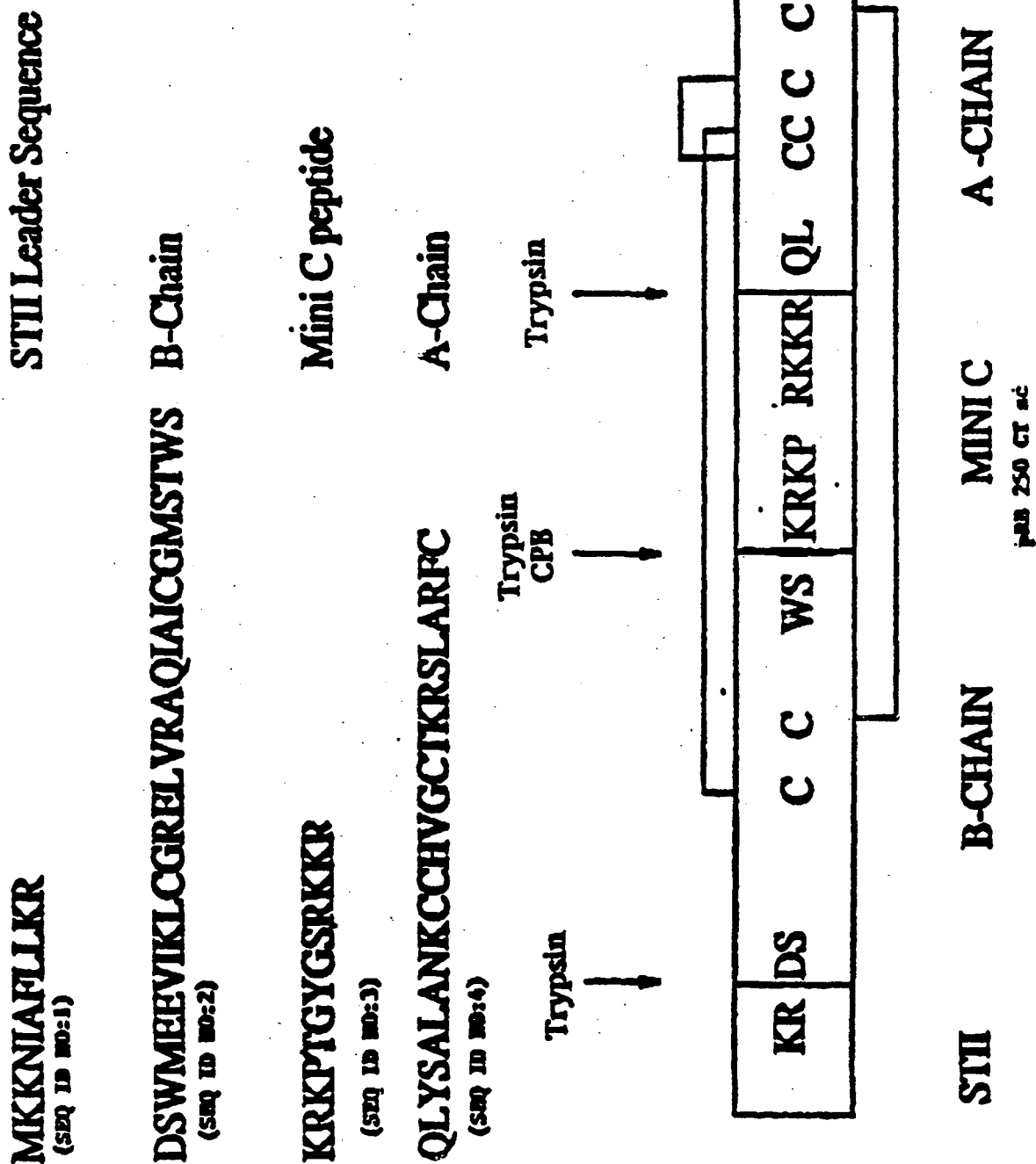
9. The process according to Claim 8 wherein said isolation includes solubilizing and refolding said prorelaxin.
10. The process of Claim 9 wherein said prorelaxin is solubilized with a solution comprising guanidine hydrochloride.
11. The process of Claim 9 wherein said prorelaxin is refolded under conditions of dilute protein concentration.
12. The process of Claim 9 wherein prorelaxin is refolded using a redox buffer.
13. The process of Claim 8 wherein said host cells are E.coli.
14. The process of Claim 1 further comprising cyclizing the A-chain N-terminal glutamine.
15. The process of Claim 14 wherein the relaxin A-chain N-terminal glutamine is cyclized through a heat step.
16. The process according to Claim 1 wherein said non-naturally occurring C-peptide is comprised of the amino acid sequence KRKPTGYGSRKKR.
17. The process according to Claim 1 wherein said non-naturally occurring C-peptide is comprised of the amino acid sequence DKKRTGYGSRRRK.
18. The process according to Claim 1 wherein said non-naturally occurring C-peptide is comprised of the amino acid sequence DKKRTGYGSRKKR.

19. The process according to Claim 1 wherein said non-naturally occurring C-peptide is comprised of the amino acid sequence KRKPTGYGSRRRK.
20. The process according to Claim 1 wherein said leader sequence is comprised of the amino acid sequence MKKNIAFLLKR.
21. The process according to Claim 1 wherein said relaxin is recovered by a process comprising use of adsorption chromatography, ion-exchange chromatography, reverse-phase chromatography, size-exclusion chromatography and/or ultrafiltration.
22. The process according to Claim 1 optionally comprising formulating the relaxin in formulation buffer.
23. A prorelaxin comprising a leader peptide, the B-chain, a non-naturally occurring C-chain, and the A-chain, wherein said leader peptide comprises a cleavage site adjacent the B-chain and wherein said non-naturally occurring C-chain comprises cleavage sites adjacent the B-chain and the A-chain.
24. The prorelaxin according to Claim 23 wherein said cleavage sites are enzymatically cleaveable.
25. The prorelaxin according to Claim 23 wherein said non-naturally occurring C-peptide is comprised of the amino acid sequence KRKPTGYGSRRKKR.
26. The prorelaxin according to Claim 23 wherein said non-naturally occurring C-peptide is comprised of the amino acid sequence DKKRTGYGSRRRK.
27. The prorelaxin according to Claim 23 wherein said non-naturally occurring C-peptide is comprised of the amino acid sequence DKKRTGYGSRRKKR.

28. The prorelaxin according to Claim 23 wherein said non-naturally occurring C-peptide is comprised of the amino acid sequence KRKPTGYGSRRRK.
29. The prorelaxin according to Claim 23 wherein said leader sequence is comprised of the amino acid sequence MKKNIAFLLKR.
30. An isolated DNA encoding the prorelaxin of Claim 23.
31. An expression vector operatively containing the DNA according to Claim 30.
32. A host cell transfected with the vector of claim 31.

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FIG. 1

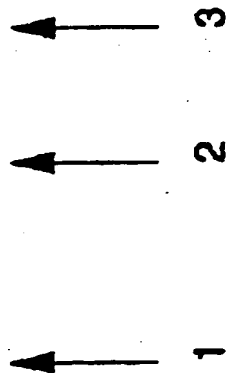


2/28

Plasmid Name	Leader Sequence	C chain sequence	Cleaving Enzymes
pR250CTac	MGQNIAFLLKR	KRKPTGYGSRARRK 5	Trypsin and carboxy-peptidase C (CPB)
pRELCH	MGQNIAFLLKR	DKKRTGYGSRARRK 6	AspN and LysC
pRELAspN	MGQNIAFLLKR	DKKRTGYGSRARRK 7	aspN and trypsin or ArgC
pREL _{LysC}	MGQNIAFLLKR	KRKPTGYGSRARRK 8	LysC and CPB

FIG. 2

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CONST	STII/B-CHAIN	BC/MC	MC/AC	SITE	ENZYMES
pRB250CTsc	↓ 1	↓ 2	↓ 3	1	TRYP.; ASP-N
	KR DS	...WS KRKP...	RKKR QL	2	TRYP.; ARG-C; LYS-C; ALL WITH CPB
				3	TRYP.; ARG-C

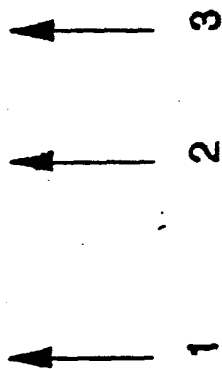
MOST FAVORABLE COMBINATIONS

ARG-C / CPB
TRYPSIN / CPB

FIG. 2A

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STII	B-CHAIN	MINI-C	A-CHAIN
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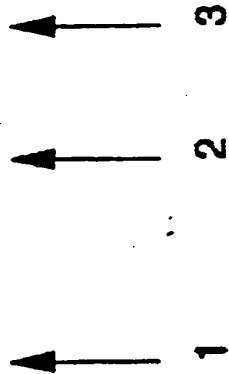


CONST	STII/B-CHAIN	BC/MC	MC/AC	SITE	ENZYMES
pRELCIII	KR DS	...WS DKKR	...RRRK Q	1	TRYP.; ASP-N
				2	ASP-N
				3	TRYP.; LYS-C

MOST FAVORABLE COMBINATIONS

ASP-N / LYS-C
ASP-N / TRYPSIN

FIG. 2B



CONST	STII/B-CHAIN	BC/MC	MC/AC	SITE	ENZYMES
pRELCLysC	↓ 1 KR DS	↓ 2 WS KRK	↓ 3 ...RRRK Q	1	TRYP.; ASP-N
				2	TRYP.; ARG-C LYS-C; ALL WITH CPB
				3	TRYP.; LYS-C

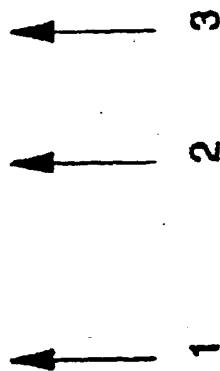
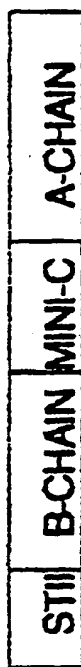
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MOST FAVORABLE COMBINATIONS

TRYPSIN / CPB
 TRYPSIN / LYS-C / CPB
 ARG-C / LYS-C / CPB

FIG. 2C

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CONST	STII/B-CHAIN	BC/MC	MC/AC	SITE	ENZYMES
pRELCaspN	↓ 1 KR DS	↓ 2 ...WS DKKR	↓ 3 ...RKRR Q	1	TRYP.; ASP-N
				2	ASP-N
				3	TRYP.; ARG-C

MOST FAVORABLE COMBINATIONS

ASP-N / ARG-C
ASP-N / TRYPSIN

FIG. 2D

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ATG AAA AAG AAT ATC GCA TTT CTT CTT AAA CGG GAC TCA 36
 Met Lys Lys Asn Ile Ala Phe Leu Leu Lys Arg Asp Ser
 1 5 10
STII leader + KB

TGG ATG GAG GAA GTT ATT AAA TTA TGC GGC CGC GAA TTA 75
 Trp Met Glu Glu Val Ile Lys Leu Cys Gly Arg Glu Leu
 15 20 25
B-chain relaxin

GTT CGC GCG CAG ATT GCC ATT TGC GGC ATG AGC ACC TCG 114
 Val Arg Ala Gln Ile Ala Ile Cys Gly Met Ser Thr Trp
 30 35

AGC AAA ACG AAA CCC ACT GGT TAT GGT TCT CGA AAA AAG 153
 Ser Lys Arg Lys Pro Thr Gly Tyr Gly Ser Arg Lys Lys
 40 45 50
Synthetic DNA Encoding C-Peptide

AGA CAA CTC TAC AGT GCA TTG GCT AAT AAA TGT TGC CAT 192
 Arg Gln Leu Tyr Ser Ala Leu Ala Asn Lys Cys Cys His
 55 60
A-chain relaxin

GTT GGT TGT ACC AAA AGA TCT CTT GCT AGA TTT TGC 228
 Val Gly Cys Thr Lys Arg Ser Leu Ala Arg Phe Cys
 65 70 75 76

FIG. 3

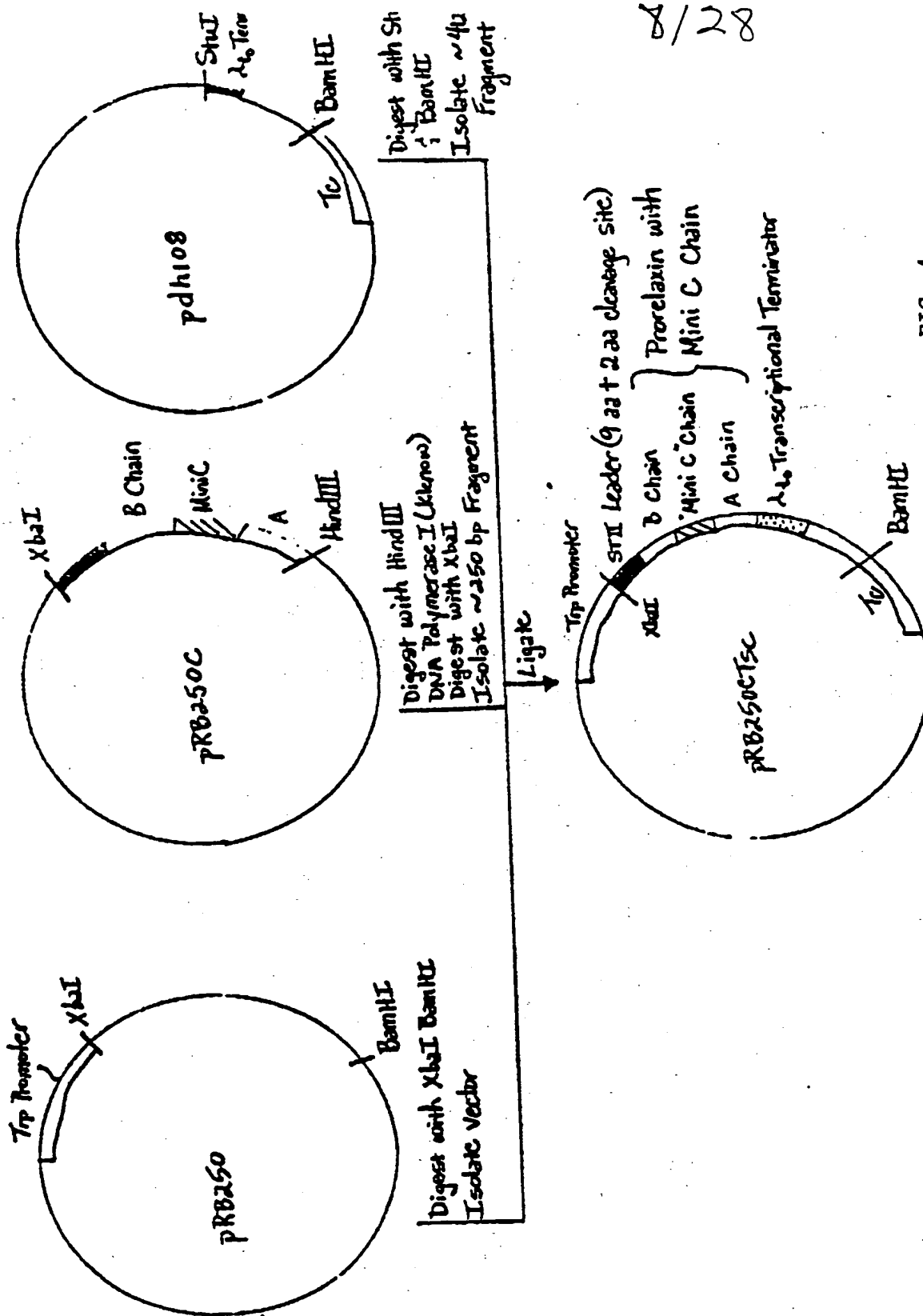


FIG. 4

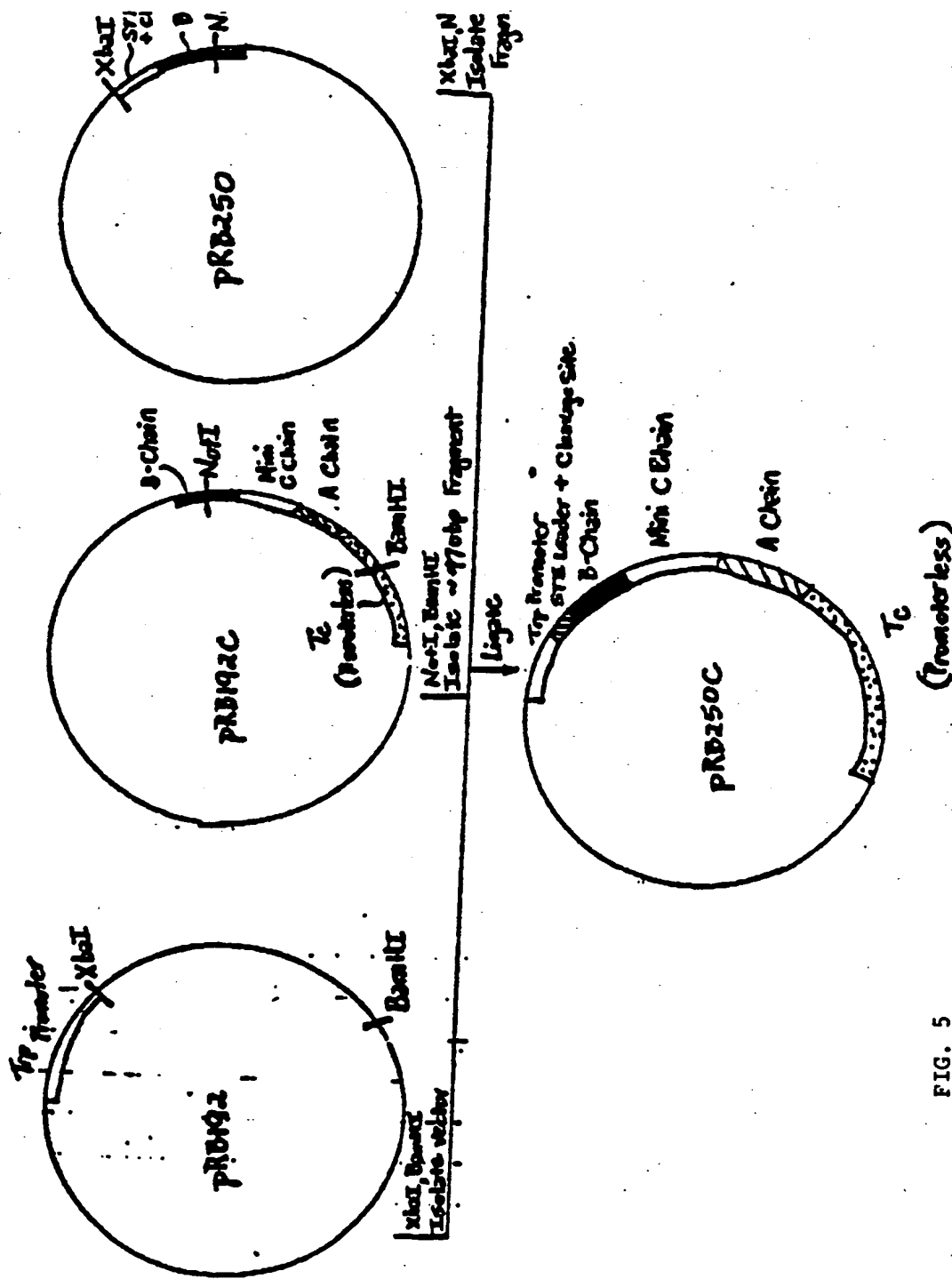


FIG. 5

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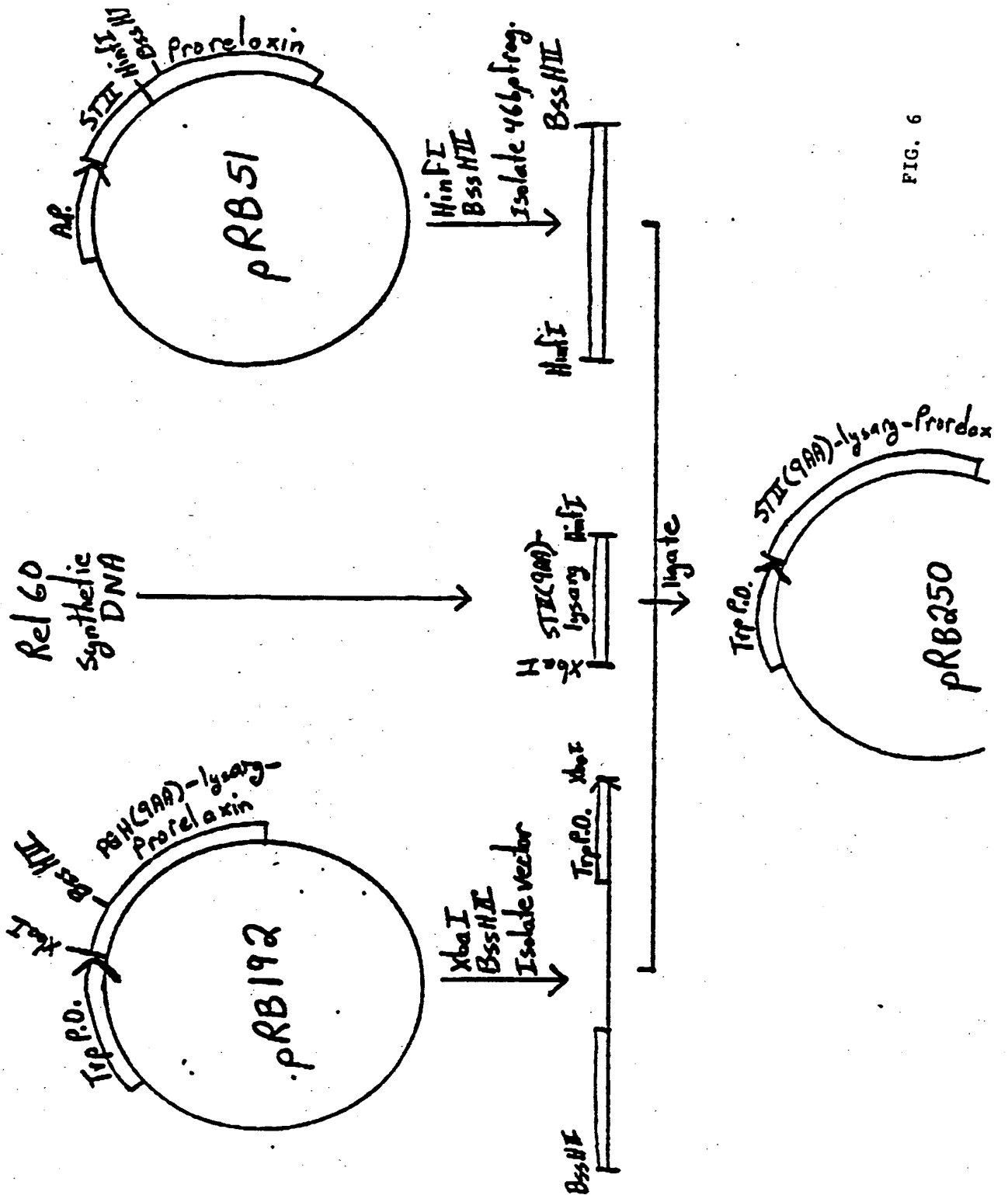


FIG. 6

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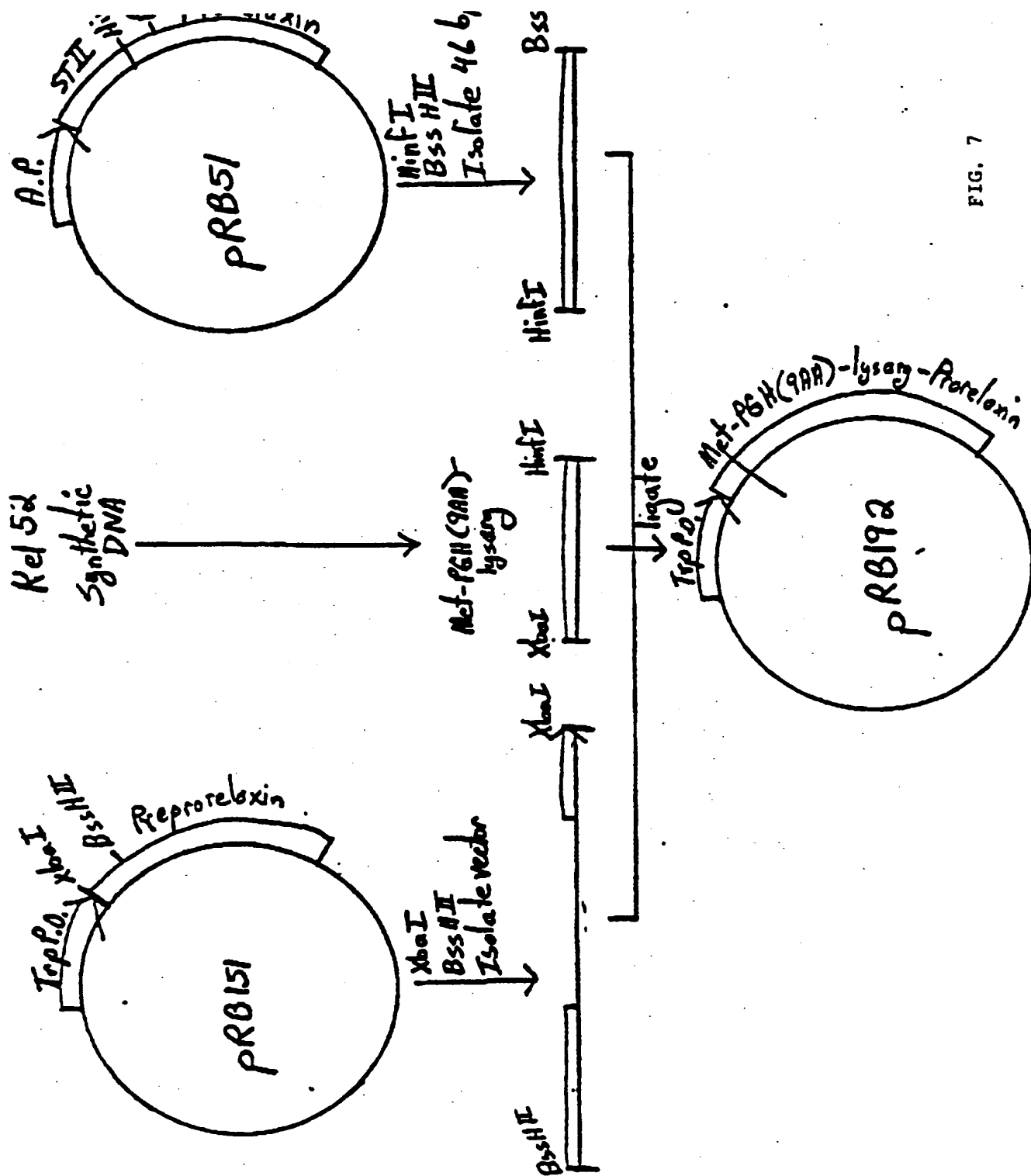


FIG. 7

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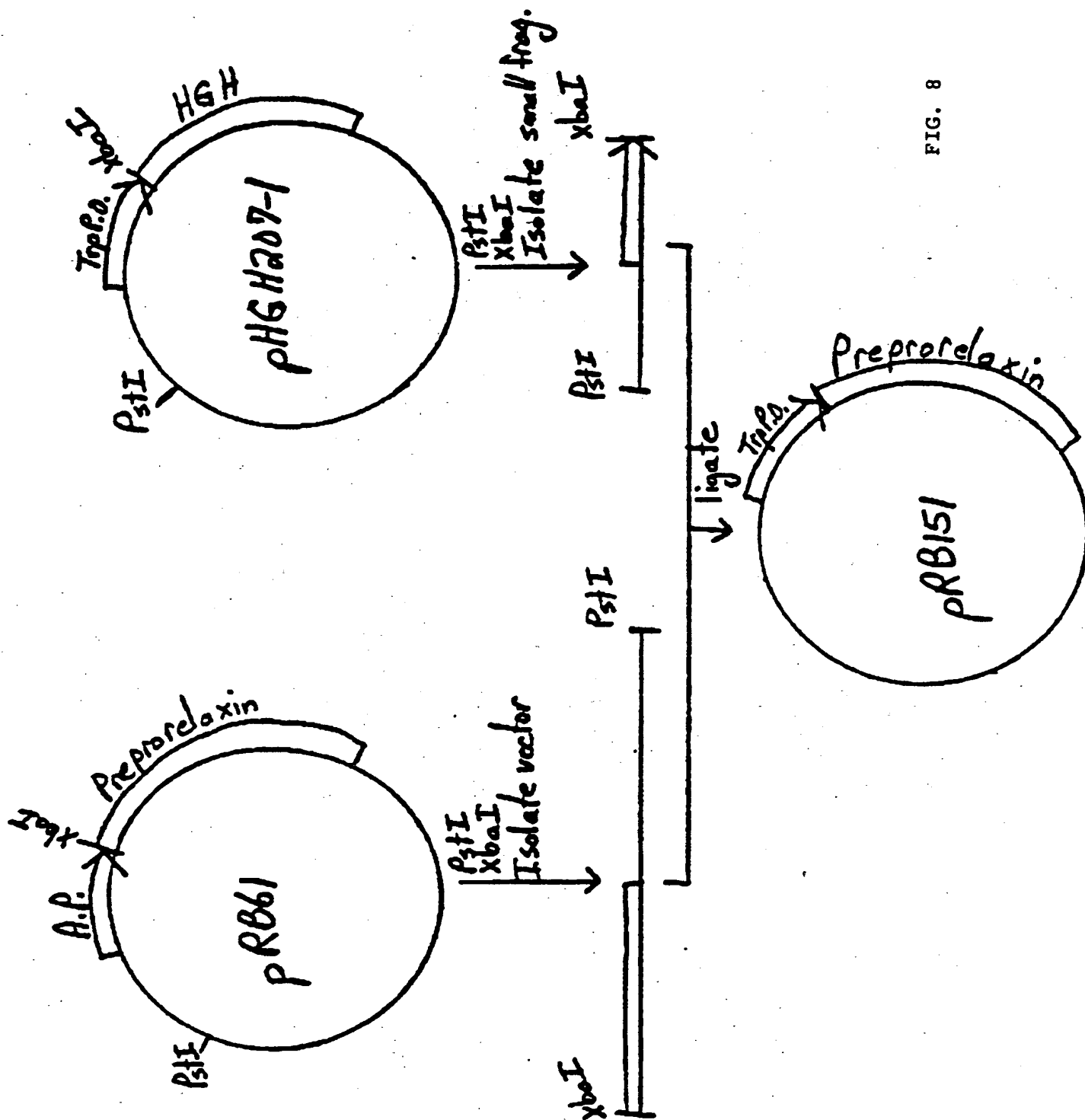


FIG. 8

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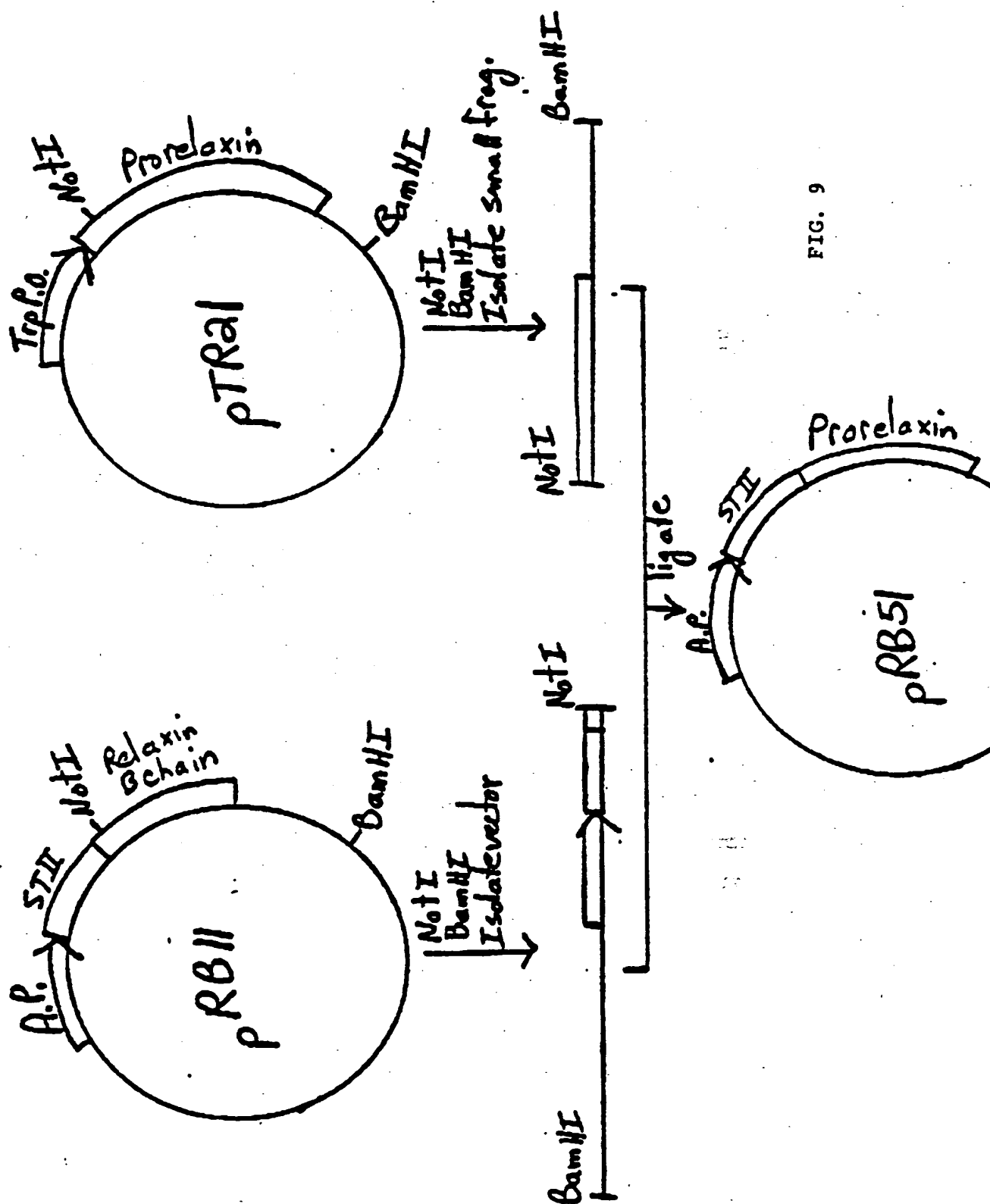


FIG. 9

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GlyArgGluLeuValArgAlaGlnIleAlaIleCysGlyMetSerThrTrpSerLysArgSerLeuSerGlnGluAsp
GCGGCCGCGAATTAGTTCGCGCGCAGATTGCCATTTGCGGCATGAGCACCTGGAGCAAAAGGTCTCTGAGCCAGGAAGAT

AlaProGlnThrProArgProValAlaGluIleValProSerPheIleAsnLysAspThrGluThrIleAsnMetMetSer
GCTCCTCAGACACCTAGACCAGTGGCAGAAATTGTGCCATCCTTCATCAACAAAGATACAGAAACCATAAATATGATGTC

GluPheValAlaAsnLeuProGlnGluLeuLysLeuThrLeuSerGluMetGlnProAlaLeuProGlnLeuGlnGlnHis
AGAATTTGTTGCTAATTTGCCACAGGAGCTGAAGTTAACCCTGTCTGAGATGCAGCCAGCATTACCACAGCTACAACAAC

ValProValLeuLysAspSerSerLeuLeuPheGluGluPheLysLysLeuIleArgAsnArgGlnSerGluAlaAla
ATGTACCTGTATTAAAAGATTCCAGTCTTCTCTTTGAAGAATTTAAGAACTTATTTCGCAATAGACAAAGTGAAGCCGCA

AspSerSerProSerGluLeuLysTyrLeuGlyLeuAspThrHisSerArgLysLysArgGlnLeuTyrSerAlaLeuAla
GACAGCAGTCCTTCAGAATTAAATACTTAGGCTTGGATACTCATTCTCGAAAAAAGAGACAACTCTACAGTGCATTGGC

AsnLysCysCysHisValGlyCysThrLysArgSerLeuAlaArgPheCys
TAATAAATGTTGCCATGTTGGTTGTACCAAAGATCTCTTGCTAGATTTTGCTGAGATGAAGCTAATTGTGCACATCTCG

TATAATATTACACATATTCTTAATGACATTTCACTGATGCTTCTATCAGGTCAATTCTCATGTTTGACAGCTTATCATC

GATAAGCTTTAATGCGGTAGTTTATCACAGTTAAATTGCTAACGCAGTCAGGCACCGTGTATGAAATCTAACAATGCGCT

CATCGTCATCCTCGGCACCGTCACCCTGGATGCTGTAGGCATAGGCTTGTTATGCCGGTACTGCCGGGCCTCTTGCGGG

ATATCGTCCATTCCGACAGCATCGCCAGTCACTATGGCGTGCTAGCGCTATATGCGTTGATGCAATTTCTATGCGCA

CCCGTTCTCGGAGCACTGTCCGACCGCTTTGGCCGCCGCCAGTCCTGCTCGCTTCGCTACTTGAGCCACTATCGACTA

CGCGATCATGGCGACCACCCCGTCTGTGGATCC

FIG. 9A

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GAATTCACCTTCCTCATACTTTGGATAAGGAATAACAGACATGAATAATCTCATTTGCTGAGTTCTTATTAAAGCTTGCCCC
 AAAAGAGAGAGAGTCCGAAGAACTGTGTGCGCAGGTAGAACCTTTGGAGATTATCGTCACCTGCATGCTTCGCCATATAG
 GCGCAAAATACCCACACAGCGGTGATTGATCAGGTAGAGCGGCGCTGTACGAGGTAAGGCCGATGCCAGCATTCCTGA
 CACGATACGGAGCTGCTGCGCGATTACGTAAAGAGTTATTGAAGCATCCCTGTCAGTAAAGTTAATCTTTTCAACA
 GCTGTCAATAAAGTTGTGTCAGCGCGGAGACTTATAGTCCGCTTTTATTATTTTAAATGTAATTTGACGCAAGTTCACGTA

bp S.D. STII S.D. -23 -20
 AAAGGGTATCTAGAGGTTGAGGTTGATTTT Met Lys Lys Asn Ile Ala Phe Leu Leu Ala Ser Met
 ATG AAA AAG AAT ATC GCA TTT CTT GCA TCT ATG

-10 -1
 Phe Val Phe Ser Ile Ala Thr Asn Ala Tyr Ala Asp Ser Trp Met Glu Glu Val Ile Lys
 TTC GTT TTT TCT AAT GCT ACA AAT GCC TAT GCA GAC TCA TGG ATG GAG GAA GTT ATT AAA

10 20
 Leu Cys Gly Arg Glu Leu Val Arg Ala Gln Ile Ala Ile Cys Gly Met Ser Thr Trp Ser
 TTA TGC GGC CGC GAA TTG GTA CCC GCG CAA ATA GCG ATA TGC GGT ATG AGT ACA TGG AGT

TGAAGA

FIG. 10

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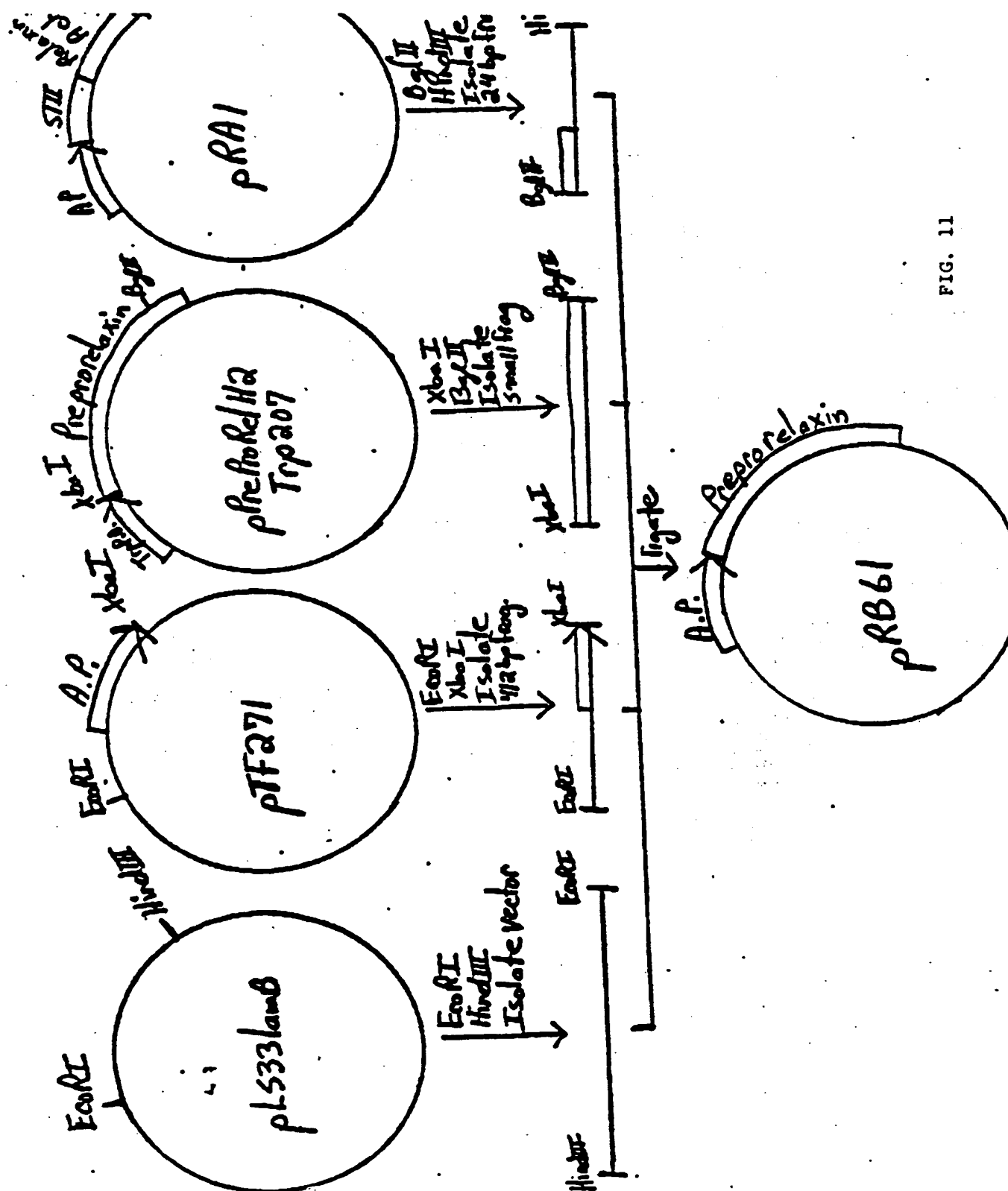


FIG. 11

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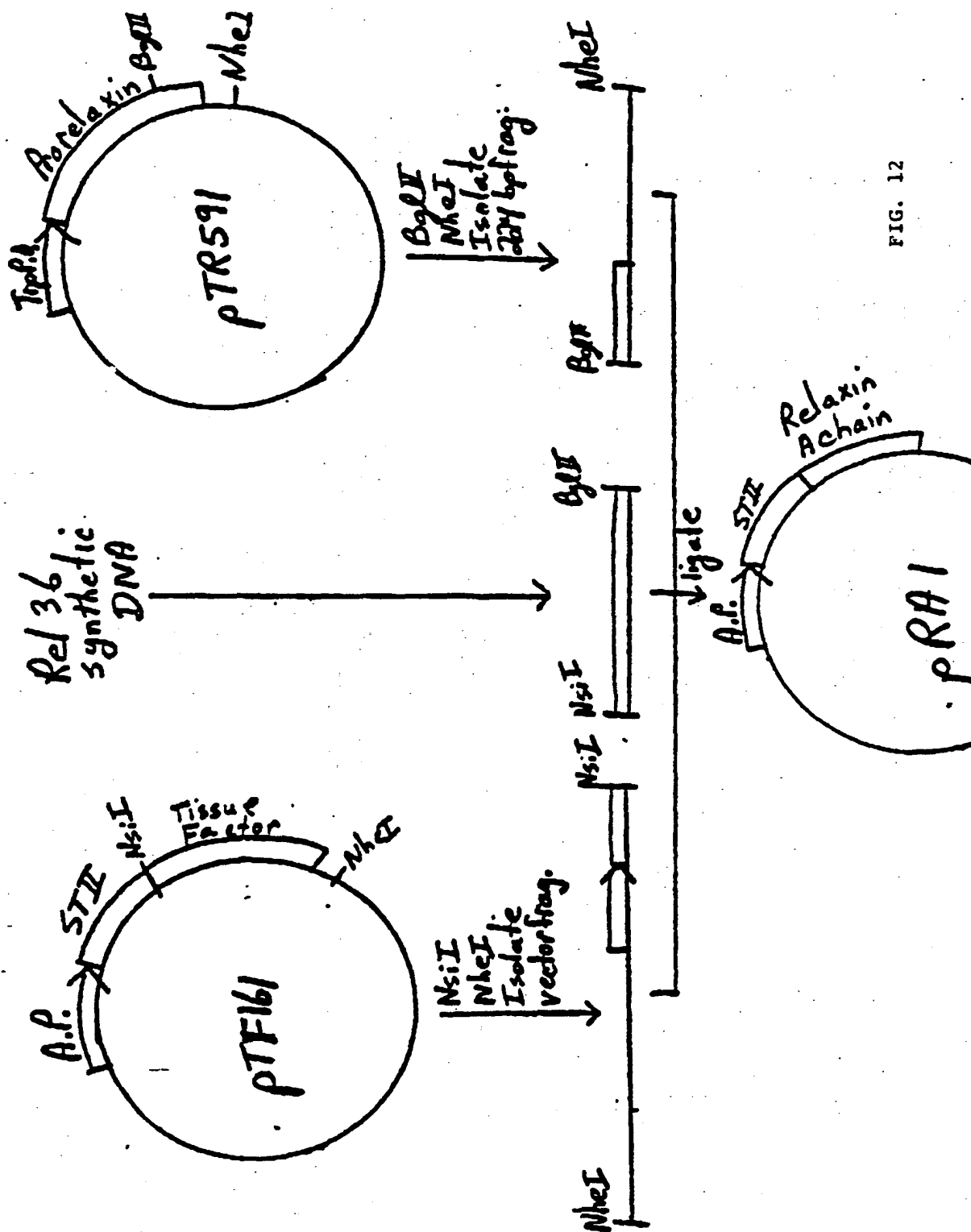


FIG. 12

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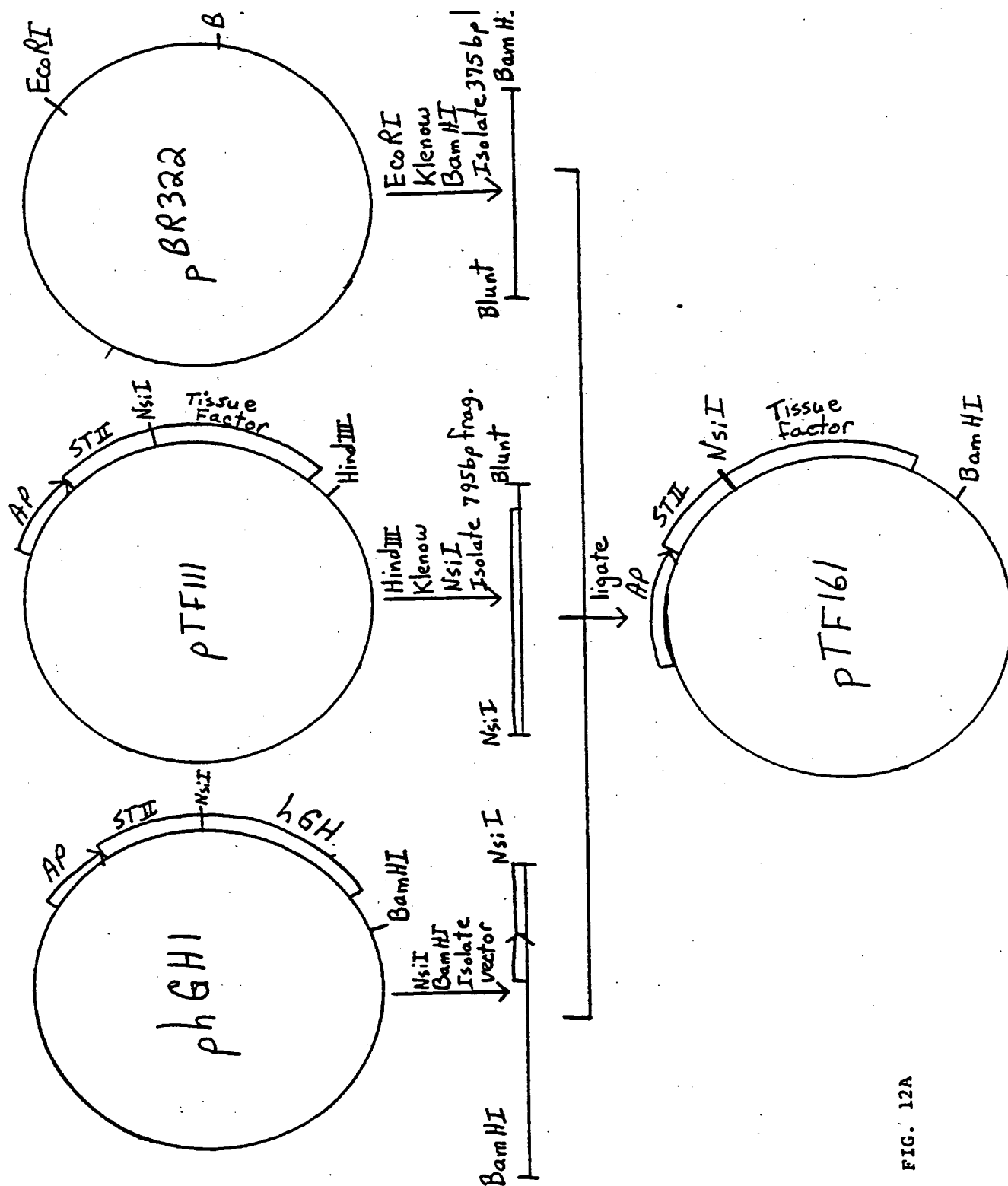


FIG. 12A

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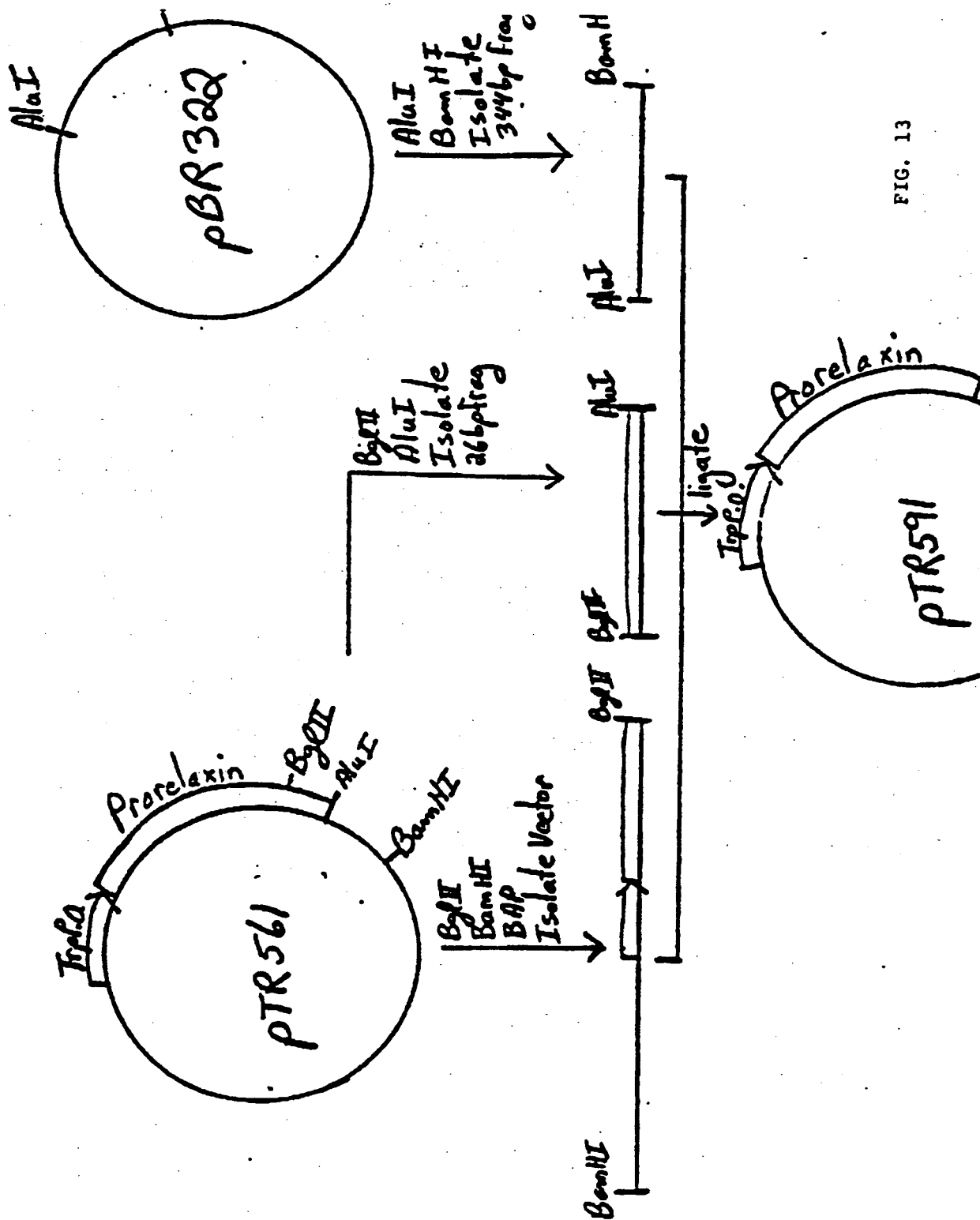


FIG. 13

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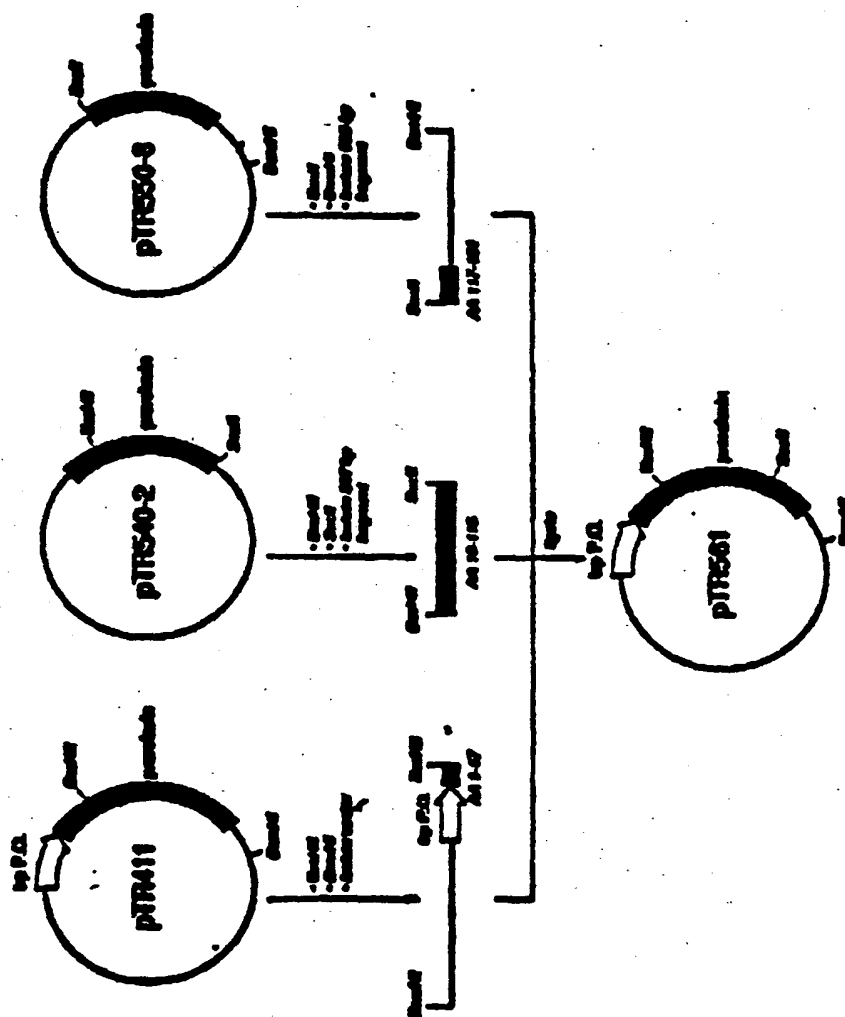


FIG. 14

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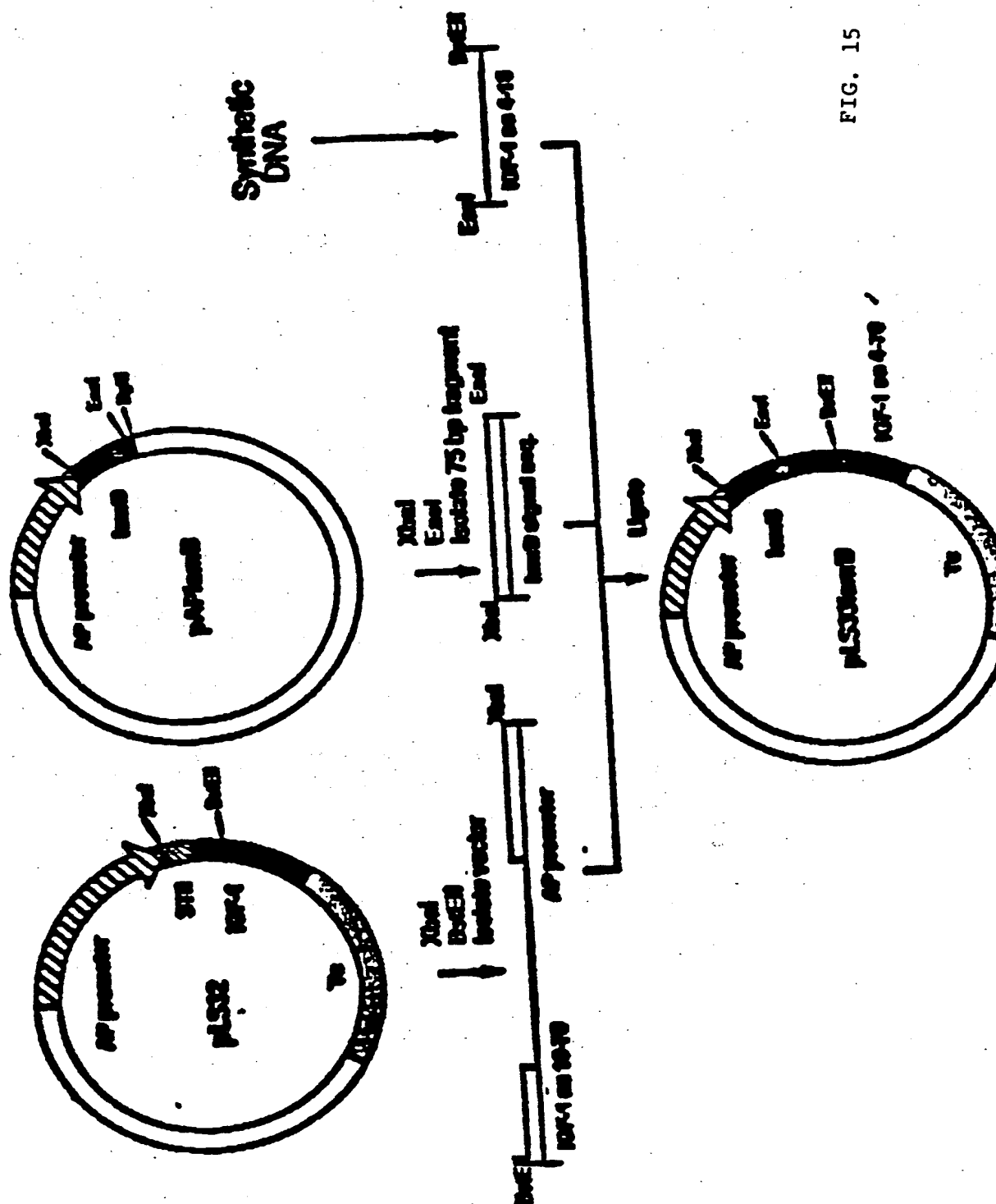


FIG. 15

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GAATTCAACTTCTCCATACTTTGGATAACGAAATACAGACATGAAAAATCTCATTTGCTGAGTTGTATTTAAGCTTGCCC
AAAAGAAGAAGAGTGCAGAAAGAACTGTGTGCGCAGGTACGAAGCTTTGGAGATTATCGTCAC TGCAATGCTTCGCAATATG
CGCGAAAAATGACCAACAGCGGTGATTGATCAGGTAGACGGGGCGCTGTACGAGGTAAAGCCCGATGCCAGCATCTTCGA
CGACGATACGGAGCTGCTCGCGGATTACGTAAGAAGTATTGAACCATCTCTGTCAGTAAAAAGTTAATCTTTCAACA
CGTGTCATAAAGTTGTCAACCGGACAGACTTATAGTCTGTTTGTGTTTATTATTTTAAATGTAATTGTAAGTACGCAAGT

WT S.D. STII S.D. -23 -20
 Met Lys Lys Asn Ile Ala Phe Leu Leu Ala
 ATG AAA AAG AAT ATC GCA TTT CTT CTT GCA

-10 ↓ 1

Ser Met Phe Val Phe Ser Ile Ala Thr Asn Ala Tyr Ala Ser Gly Thr Thr Asn Thr Val
TCT ATG TTC GTT TTT TCT ATT GCT ACA AAT GCC TAT GCA TCA GGC ACT ACA AAT ACT GTG

20 20

Ala	Ala	Tyr	Asn	Leu	Thr	Trp	Lys	Ser	Thr	Asn	Phe	Lys	Thr	Ile	Leu	Glu	Trp	Glu	Pro
GCA	GCA	TAT	AAT	TTA	ACT	TGC	AAA	TCA	ACT	AAT	TTC	AAC	ACA	ATT	TTG	GAG	TGG	GAA	CCC

30
 Lys Pro Val Asn Gln Val Tyr Thr Val Gln Ile Ser Thr Lys Ser Gly Asp Trp Lys Ser
 AAA CCC GTC AAT CAA GTC TAC ACT GTT CAA ATA ACC ACT AAG TCA GGA GAT TCG AAA AGC

Lys Cys 50 60 Lys Cys
 AAA TGC Phe Tyr Thr Thr Asp Thr Glu Cys Asp Leu Thr Asp Glu Ile Val Lys Asp Val
 TTT TAC ACA ACA GAC ACA GAG TGT GAC CTC ACC GAC GAG ATT GTC AAG GAT GTG

70
Lys Gln Thr Tyr Leu Ala Arg Val Phe Ser Tyr Pro Ala Gly Asn Val Glu Ser Thr Gly
AAG CAG ACG TAC TTG GCA CGG GTC TTC TCC TAC CCG GCA GGG AAT GTG GAG AGC ACC GGT

90 100

Ser Ala Gly Glu Pro Leu Tyr Glu Asn Ser Pro Glu Phe Thr Pro Tyr Leu Glu Thr Asn
TCT GCT GGG GAG CCT CTG TAT GAG AAC TCC CCA GAG TTC ACA CCT TAC CTC GAG ACA AAC

110 120
Leu Gly Gln Pro Thr Ile Gln Ser Phe Glu Gln Val Gly Thr Lys Val Asn Val Thr Val
CTC GCG CAG CCA ACC ATT CAG ACT TTT CAA CAG GTC GGA ACA AAA GTG AAC GTG ACC GTA

130 140

Glu Asp Glu Arg Thr Leu Val Arg Arg Asn Asn Thr Phe Leu Ser Leu Arg Asp Val Phe
GAG GAT GAA GCG AGC TTA CTC ACC ACC AAC AAC ACC TTT CTC CTA ACC CTC GCG GAT GTT TTT

150

Gly	Lys	Asp	Leu	Ile	Tyr	Thr	Leu	Tyr	Tyr	Trp	Lys	Ser	Ser	Ser	Gly	Lys	Lys	Thr	
CCT	AAA	GAC	TAA	ATT	TCA	AAC	TTT	TAT	TAT	TGG	AAA	TCT	TCA	AGT	TCA	GGA	AAG	AAA	ACA

160

Gly	Lys	Asp	Leu	Ile	Tyr	Thr	Leu	Tyr	Tyr	Trp	Lys	Ser	Ser	Ser	Gly	Lys	Lys	Thr	
CCT	AAA	GAC	TAA	ATT	TCA	AAC	TTT	TAT	TAT	TGG	AAA	TCT	TCA	AGT	TCA	GGA	AAG	AAA	ACA

170
Ala Lys Thr Asn Thr Asn Glu Phe Leu Ile Asp Val Asp Lys Gly Glu Asn Tyr Cys Phe
180
TTC TGG CAG TGC CAG CAG ACC GCG GAG AAC TAC TGT TTC

180										200									
Ser	Val	Gln	Ala	Val	Ile	Pro	Ser	Arg	Thr	Val	Asn	Arg	Lys	Ser	Thr	Asp	Ser	Pro	Val
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40
41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60
61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80
81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100

220
Glu Cys Met Gly Gln Glu Lys Gly Glu Phe Arg Glu Ile Phe Tyr Ile Ile Gly Ala Val

Val Phe Val Val Ile Ile Leu Val Ile Ile Leu Ala Ile Ser Leu His OC₂

TTGACAGCTTATCATCGATAACCTTTAATCGGTAATTTATCAGCTTAAATTGCTAACGCAGTCAGGCACTGGTAT
AATCTAACAATCGCTCATCGTCATCTTCGGCACCGTCACCGTGGATGCTGTAGGCATAGGCTTGTTATGCCCGTAC
CCGGCCCTCTTGGGGATATCGTCCATTCCGACAGATCGCCAGTCACTATGGCGTGCTGCTAGCGCTATATCGCTTG

FIG. 16

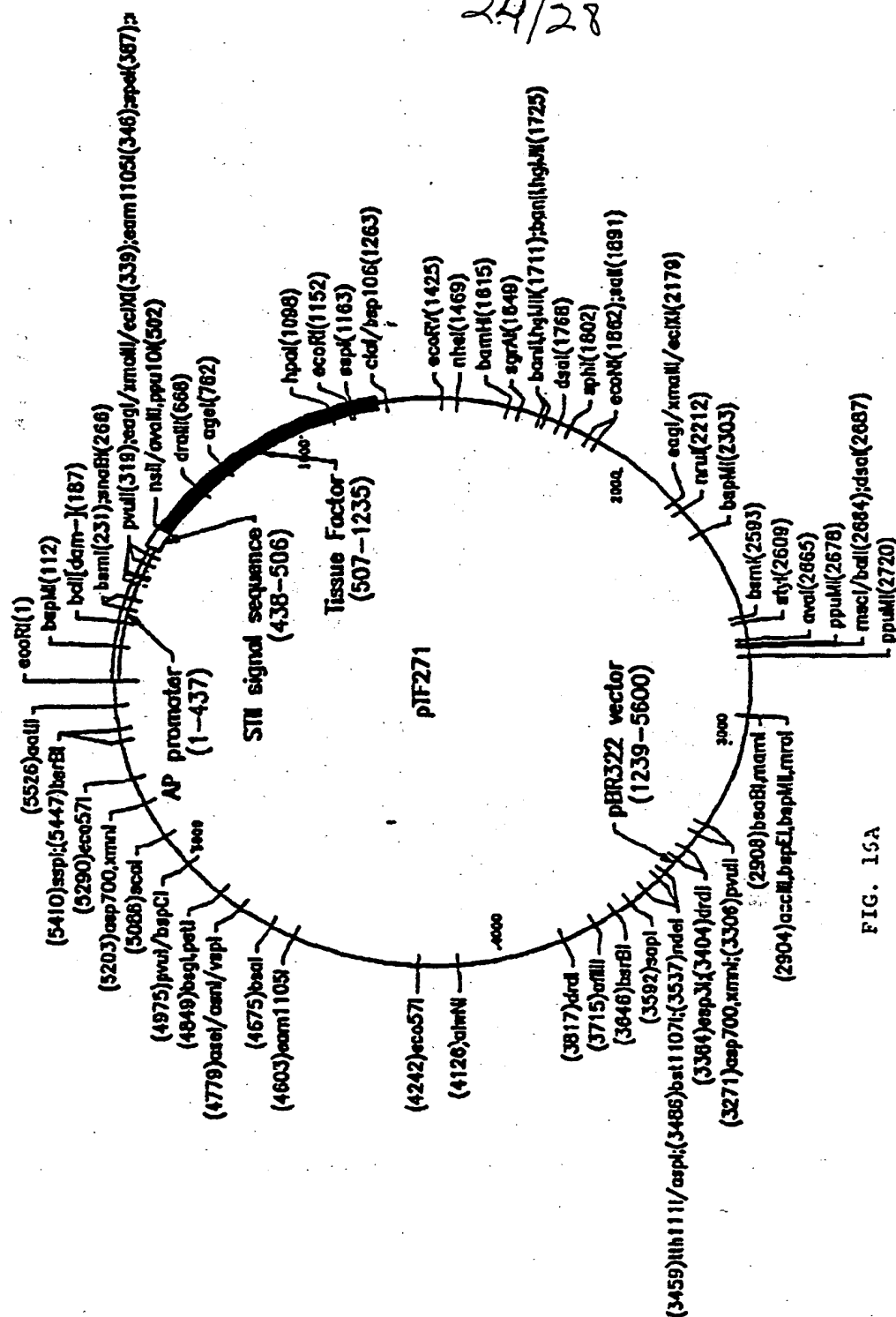


FIG. 15A

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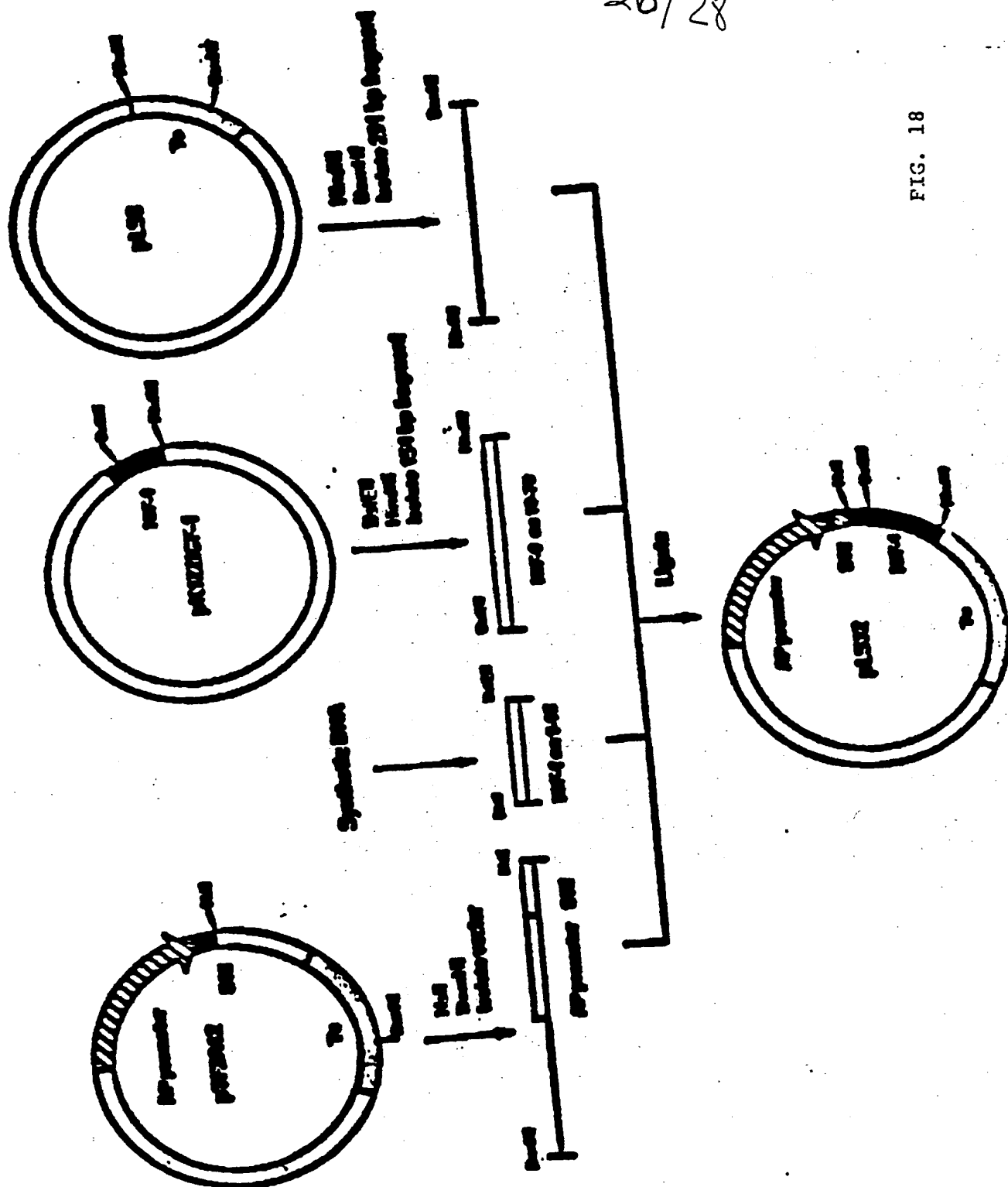


FIG. 18

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MetLysSerAsnAsnAlaLeuIleValIleLeuGlyThrValThrLeuAspAlaValGlyIleGlyLeuValMet
AAGCTTATGAAATCTAACAAATGCGCTCATCGTCATCCTCGGCACCGTCACCCCTGGATGCTGTAGGCATAGGCTTGGTTAT

ProValLeuProGlyLeuLeuArgAspIleValHisSerAspSerIleAlaSerHisTyrGlyValLeuLeuAlaLeuTyr
GCCGGTACTGCCGGGCCCTCTTGGGGATATCGTCATTCGACAGCATCGCCAGTCACTATGGCGTGCTGTAGCGCTAT

AlaLeuMetGlnPheLeuCysAlaProValLeuGlyAlaLeuSerAspArgPheGlyArgArgProValLeuAla
ATCGGTTGATGCAATTTCTATGCGCACCCGTTCTCGGAGCACTGTCCGACCGCTTGGCCGCCAGTCTGCTCGCT

SerLeuLeuGlyAlaThrIleAspTyrAlaIleMetAlaThrThrProValLeuTrp
TCGCTACTTGGAGCCCACTATCGACTACGGCATCATGGCGACCACACCCGTCCTGTGGATCC

FIG. 18A

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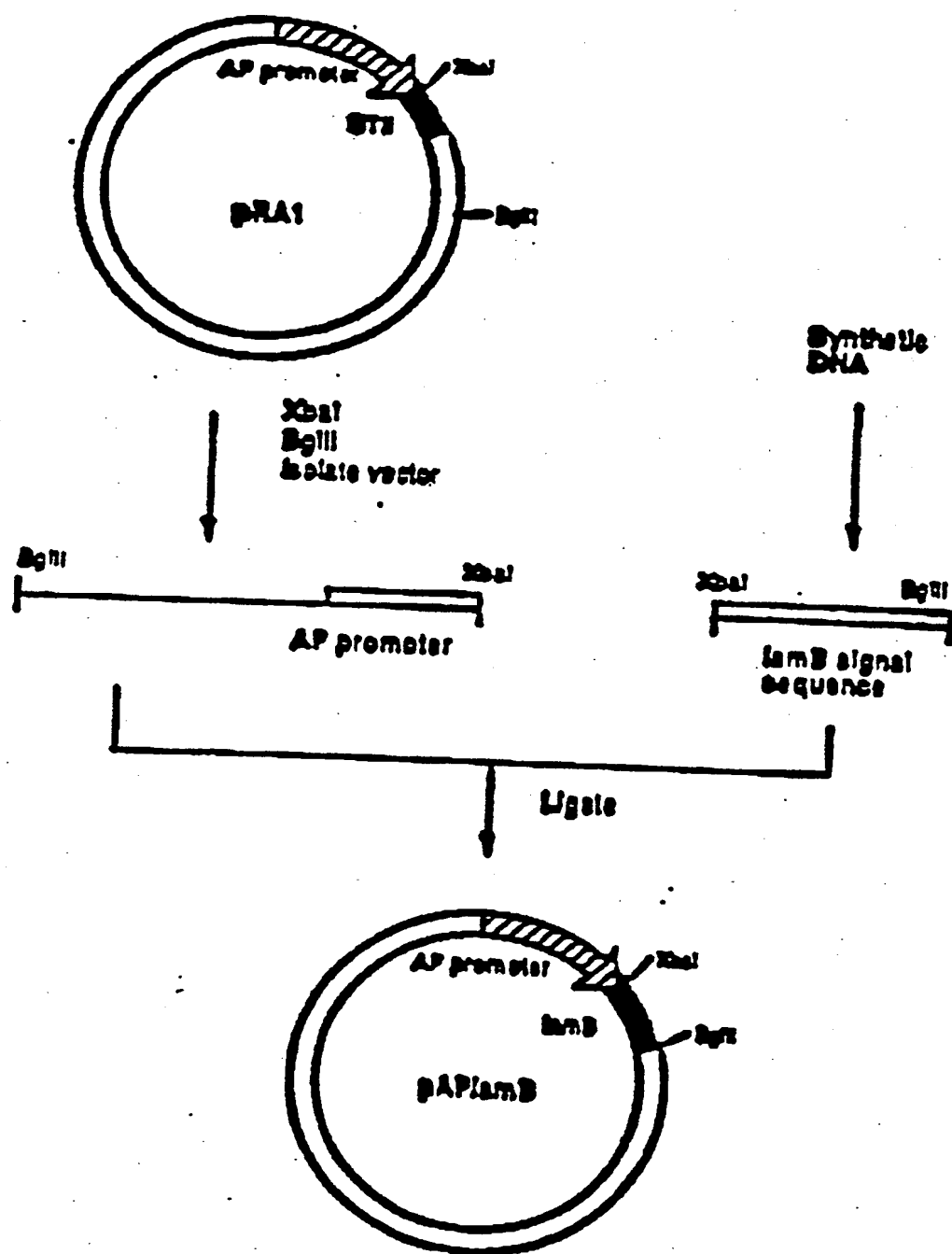


FIG. 19

